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PRINCIPAL INVESTIGATOR: Robert E. Burke, M.D.

CONTRACTING ORGANIZATION: Columbia University
New York, NY 10032

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14. ABSTRACT The molecular processes of programmed cell death (PCD) are important mediators of neural degeneration in Parkinson's disease (PD). The goal of this proposal is to examine in living animals the possible role of ER stress, a mediator of PCD, in dopamine neuron death. This is being done by the study of mice with targeted deletions of CHOP and caspase-12, mediators of ER stress-induced apoptosis. We have demonstrated that CHOP is universally expressed in neurotoxin models of parkinsonism. Assessment of the functional significance of CHOP expression by study of CHOP null mice has shown that in the adult 6OHDA model there is diminished apoptosis. The null mutation does not, however, protect dopamine neurons in the chronic MPTP model. We therefore conclude that CHOP is expressed and uniquely plays a functional role in the adult 6OHDA model. It may do so either in response to ER stress, or to oxidative stress. Since our last progress report, we have completed our studies of caspase-12 mice in the adult 6OHDA model. These studies have shown that caspase-12 mice are not protected from 6OHDA; they do not show a diminished level of apoptosis, and they do not show an increased survival of dopaminergic neurons. Since caspase-12 is a proven critical mediator of PCD due to ER stress, these results would suggest that the upregulation of CHOP in the 6OHDA model is not mediated by ER stress, but rather oxidative insult. In the final year of this award, we intend to determine where CHOP acts in the molecular pathways of PCD in relation to signaling by c-jun phosphorylation. This will be done by examining the effect on CHOP expression of null mutations in both JNK2 and JNK3.

15. SUBJECT TERMS
apoptosis, programmed cell death, 6-hydroxydopamine, MPTP Parkinson disease

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Table of Contents

INTRODUCTION

There is a growing consensus that the molecular processes of programmed cell death (PCD) are important mediators of neural degeneration in Parkinson's disease (PD) and related disorders. However, while important recent advances in PD research have implicated both environmental and genetic factors in the pathogenesis of the disease, it has been unclear how these factors initiate the PCD cascade. The recent advances in our understanding of the genetic basis of PD, related to synuclein mutations which foster protein aggregation, and parkin mutations which result in a loss of functional ability to ubiquitinate difficult-to-fold proteins, have suggested a possible role for endoplasmic reticulum (ER) stress. In addition, it has been shown by analysis of gene expression in neurotoxin models in tissue culture, that ER stress may play a role in the PCD of dopamine neurons (1,2). The goals of this proposal are to examine in living animals whether CHOP, an upstream transcriptional mediator of ER stress-induced apoptosis, and caspase-12, a downstream mediator, play a role in PCD of dopamine neurons in neurotoxin models of parkinsonism. These goals will be achieved by studying mice with null mutations for these mediators. The sensitivity of the null animals to the induction of apoptosis in dopamine neurons will be examined in well-characterized and validated models of parkinsonism: intrastriatal injection of 6-hydroxydopamine in immature and adult mice, and chronic, systemic injection of MPTP in mice. Since the original submission of this proposal, we have made an important observation relevant to the molecular basis of PCD in dopamine neurons which leads us to expand upon our original goals. We have determined that the two kinases which phosphorylate the N-terminal of the transcription factor c-jun (JNK2 and JNK3) are essential mediators of PCD in dopamine neurons: in mice with combined JNK2/JNK3 null mutations, there is a complete abrogation of PCD induced by 6OHDA. Given our demonstration in the work supported thus far by this proposal that CHOP is also an essential mediator of PCD, we are now led to ask, where does CHOP lie in the molecular pathways of PCD in relation to JNK2 and JNK3? We will now address this question by examination of the expression of CHOP in mice null for JNK2, JNK3 and both JNK2/3, following 6OHDA administration.

BODY

In our original proposal, we submitted preliminary data which indicated that ER stress is likely to occur and to be a mediator of programmed cell death (PCD) in neurotoxin models of parkinsonism. Our colleague and collaborator on this proposal, Dr Lloyd Greene, had shown that the neurotoxin 6-hydroxydopamine (6OHDA) induces the expression of a number of mediators of an ER stress response in PC12 cells: ATF4, CHOP, BiP, phosphorylated PERK and others (1). A similar induction was noted on treatment of the cells with MPTP. He demonstrated that the ER stress response was likely to be mediating cell death in this culture model because sympathetic ganglion neurons derived from mice null for PERK, a mediator of a protective pathway in ER stress, were more sensitive to 6OHDA (1). Very similar findings were reported by Holtz and O'Malley for MN9D cells (2). The critical question which we therefore sought to address in this proposal is whether ER stress occurs in these neurotoxin models *in vivo*, and if so, whether it plays a role in mediating PCD.

On the basis of these preliminary observations, we proposed three tasks to delineate the functional roles of CHOP and caspase-12, a downstream mediator of PCD in ER stress, in dopaminergic neurotoxin-induced PCD in living animal models. We proposed to do this by studying the effects of null mutations for these mediators on dopaminergic cell death induced by 6OHDA and MPTP.

***Task 1*. To determine if CHOP is a mediator of 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**

The work for this task has now been completed, and has been published in the *Journal of Neurochemistry*. We have appended to this Annual Progress Report a copy of the published manuscript. All of the work completed for this task is presented in this full manuscript.

***Task 2*. To determine if CHOP expression is a general feature of neurotoxin-induced apoptosis in dopamine neurons of the substantia nigra *in vivo*.**

In relation to Task 2, we report in the manuscript that CHOP induction is a general feature specifically of neurotoxin-induced apoptosis in dopamine neurons, but it is not a general feature of all apoptosis in dopamine neurons. CHOP protein expression is observed in all of the 6OHDA and MPTP models tested, but it is not observed in apoptosis due to natural cell death, or that due to induction of natural cell death by axotomy (Figure 3 in the manuscript).

In the past funding period, we have shown that CHOP null mice treated with MPTP demonstrate equal levels of apoptosis as those observed in wildtype mice, and they show an equal degree of dopamine neuron loss. Thus, we have shown that there are fundamental differences in mechanisms of toxicity in the 6OHDA and MPTP models insofar as CHOP is concerned. To our knowledge, this is the first demonstration of a clear difference in mechanisms between these two models *in vivo*. This finding, however, is in keeping with the *in vitro* observations of Holtz and O'Malley, who noted a much more robust induction of CHOP, and a broader ER stress response in the 6OHDA model in comparison to the MPTP model.

Studies undertaken in response to Reviewer's comments

Our original proposal received a very fair and thorough review, and we decided that it is important to address one issue raised by the Reviewers. It was pointed out that expression of CHOP alone is not definitive evidence for the occurrence of an ER stress response. CHOP induction can occur under circumstances of oxidative stress and amino acid starvation, for example. The Reviewer therefore recommended that we examine other indicators of ER stress in our models. We selected two. One is the BiP chaperone protein, which is often (but not always) upregulated in ER stress. The other is the ER stress splice variant of the transcription factor XBP-1, which is generally considered to be the most specific indicator of ER stress (personal communications, Drs David Ron; Kazutoshi Mori).

We therefore examined the expression of BIP mRNA by Northern analysis in the chronic MPTP model at two time points: post-injection days 0 and 2. At neither time was BiP expression increased. In the adult 6OHDA model, we examined BiP expression by non-radioactive in situ hybridization. No induction was observed at 48 hours postlesion. Therefore there is no induction of this ER stress marker.

We also determined whether the XBP-1 422 bp splice variant could be identified in SN tissues in the acute or chronic MPTP or adult 6OHDA models. For this assessment, we used RNA derived from the kidney of a mouse treated with tunicamycin as a positive control. In the presence of this positive control, the XBP-1 splice variant was not detected in the chronic or acute MPTP models, or the 6OHDA model.

There are two possible conclusions. It remains possible that ER stress is occurring in these models, but these markers thereof remain below the limit of detection in these studies conducted at the tissue (as opposed to cellular) level. The second possible conclusion is that CHOP is induced in these models not on the basis of ER stress, but rather some other cellular stress, such as oxidative stress.

Task 3. **To determine if caspase-12 is a mediator of 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**

During the past year we have expanded our caspase-12 null colony, and we now have completed experiments to study the effect of the null mutation on sensitivity to death in the adult 6OHDA model. We have found that the null mutation does not reduce levels of apoptosis induced by 6OHDA: Wildtype mice: 4.1 ± 1.3 (apoptotic profiles/SN); Caspase-12 Null: 2.8 ± 1.0 (NS). In addition, we have found that the null mutation does not result in an increased survival of SN dopamine neurons. When examined at 4 weeks post 6OHDA lesion, the numbers of dopamine neurons determined by stereology are not different between the wildtype and null mice: Wild type: 4538 ± 110 (Tyrosine Hydroxylase-positive neurons/SN) (Control, Non-Lesioned side) and 1394 ± 263 (Experimental, 6OHDA-lesioned side) (a reduction of 69%); Null: 4533 ± 92 (Control) and 1350 ± 239 (Experimental, 6OHDA-lesioned side) (a reduction of 70%). Since there is substantial evidence that caspase-12 is a critical downstream mediator of PCD due to ER stress in rodent, these results would suggest that the expression of CHOP in the 6OHDA model is not mediated by ER stress, but rather oxidative stress. This conclusion is compatible with the results summarized above that other markers of ER stress were not identified in the 6OHDA model.

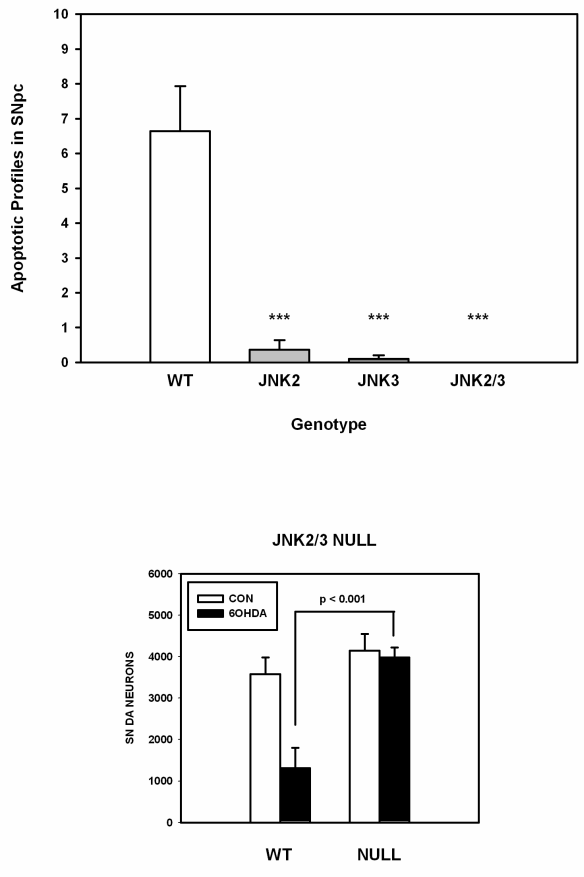

FIGURE. The effect of JNK null mutations on levels of apoptosis in SN dopamine neurons induced by 6OHDA. **A.** Single JNK2 and JNK3 null mutations result in a highly significant reduction of apoptosis following 6OHDA. The combined JNK 2/3 null mutation completely abrogates apoptosis. **B**. The abrogation of apoptosis by the combined JNK 2/3 null mutation results in a lasting protection of SN dopamine neurons. At 4 weeks following 6OHDA, there is no loss of these neurons. The difference in remaining TH-positive neuron numbers in Wildtype (WT) as compared to double-null mice is highly significant ($p<0.001$).

New Task. **To determine the molecular order of CHOP and JNK activation in 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**

In separate work, funded by our Udall Parkinson's Disease Research Center of Excellence at Columbia University, we have found that the two c-jun N-terminal kinases, JNK2 and JNK3, are essential for PCD of dopamine neurons in the 6OHDA model. As shown in the Figure, the combined JNK 2/3 double null mutation results in a complete abrogation of apoptosis, and a complete protection of these neurons. In relation to the work performed in this proposal, this result was unexpected because up to this time, there

has been no postulated upstream control of CHOP by the JNK/c-jun kinase cascade, and so we anticipated that the cell death mediated by CHOP would persist in the JNK 2/3 null mice. Since this did not occur, we are now led to ask, is JNK/c-jun activation upstream to CHOP activation? In this new Task, we will answer this question by determining the effect of the double JNK 2/3 null mutation on CHOP expression in the 6OHDA model. This will be done by performing immunohistochemistry for CHOP, as shown in the *Journal of Neurochemistry* manuscript, Figure 3.

KEY RESEARCH ACCOMPLISHMENTS

-We have demonstrated that the transcription factor CHOP, a mediator of ER stress-induced apoptosis, is expressed in the most important neurotoxin models of parkinsonism: 6OHDA-induced apoptosis in postnatal and mature rats and mice, and MPTP-induced cell death following acute or chronic administration.

-We have demonstrated that CHOP plays an essential functional role in the adult model of 6OHDA-induced apoptosis. However, we have also found that CHOP does not play a role as a mediator of neuron death in a postnatal model of 6OHDA-induced death, because in that model death is mediated primarily by an axotomy effect.

-We have demonstrated that although CHOP is expressed in both the acute and chronic MPTP models, it does not play a role as a critical mediator of neuron death, because mice null for CHOP are not protected. Thus, we have demonstrated that there is an important fundamental difference between the adult 6OHDA and MPTP models of Parkinson disease: CHOP mediates death in the former, but not in the latter. We do not know at this time which, if either, of these models is closely related to death processes in human PD. However, this demonstrated difference in these models will provide a useful basis on which to evaluate them, as we learn more about the biochemical correlates of cell death in the human disease.

-In spite of the expression of CHOP in these models, there is no further evidence at the tissue level for the expression of other markers of ER stress, including BiP and the 422 bp splice variant of the transcription factor XBP-1. It is therefore possible that CHOP expression is due to oxidative stress rather than ER stress in these models. Alternatively, it is possible that these studies, conducted at the tissue level, lacked the sensitivity to detect changes at the cellular level. Future investigations will depend on the creation of reagents which will make possible detection of these markers at the cellular level.

-The caspase-12 homozygous null mutation does not protect from 6OHDA-induced neuron death. Since caspase-12 is an important mediator of PCD induced by ER stress, we conclude that the expression of CHOP in the 6OHDA model may not be due to ER stress, as also suggested by our inability to identify other ER stress markers, but rather to an oxidative stress.

-Although we have demonstrated that CHOP is an essential mediator of PCD in the adult 6OHDA model, double null mutations of the c-jun kinases JNK2 and JNK3 completely abrogate all apoptosis in this model. This result suggests that there is a heretofore unrecognized upstream control of CHOP by these kinases. This possibility will be explored in this final year of funding.

REPORTABLE OUTCOMES FUNDED BY THIS AWARD

Silva RM, Oo TF, Jackson-Lewis VJ, Ryu E, Ron D, Przedborski S, Greene LA, Burke RE. The dopaminergic neurotoxins 6-hydroxydopamine (6-OHDA) and MPTP induce expression of CHOP (GADD153) in substantia nigra (SN) in vivo. Abstract, Society for Neuroscience, 2003.

Silva RM, Ries V, Oo TF, Yarygina O, Jackson-Lewis V, Ryu EJ, Lu PD, Stefan M. Marciniak, Ron D, Przedborski S, Kholodilov N, Greene LA, Burke RE. CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an *in vivo* neurotoxin model of parkinsonism. J Neurochemistry, 2005, 95:974-986.

Silva R, Ries V, Oo TF, Kholodilov N, Yarygina O, Jackson-Lewis V, Ryu E, Ron D, Przedborski S, Greene L, Burke RE. CHOP/Gadd153 Is A Mediator Of Apoptotic Death In Dopamine (DA) Neurons Of The Substantia Nigra (SN) In A Neurotoxin Model Of Parkinsonism. Sixth Annual Meeting of the NINDS Udall Centers for Parkinson Research, 2004.

Burke RE. Ontogenic cell death in the nigrostriatal system. In: Unsicker K (Ed). The Dopaminergic Nigrostriatal System: Development, Physiology, Disease. Cell and Tissue Research. 2004, 318:63-72. (The work done within this project on the role of CHOP in natural cell death in SN dopamine neurons was cited in this review. Support by this award is acknowledged).

Silva RM, Kuan C-Y, Rakic P, Burke RE. The Mixed Lineage Kinase-c-Jun N-Terminal Kinase Signaling Pathway: A New Therapeutic Target in Parkinson's Disease. *Movement Disorders*, 2005, 20:653-664.

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CONCLUSIONS

Based on our studies thus far, we firmly conclude that CHOP, a mediator of apoptosis due to ER stress, is upregulated in virtually all of the major neurotoxin models of parkinsonism. Our evidence indicates that CHOP is a functional mediator of apoptosis in the adult 6OHDA-induced model of parkinsonism. During this funding period, we have supported this conclusion by showing that CHOP null mice have a diminished level of apoptosis and an increased number of surviving dopamine neurons in the 6OHDA model.

Our results in the MPTP model indicate that CHOP is not a mediator of cell death in this model, in spite of the fact that it is robustly upregulated. During this funding period, we have supported this conclusion by showing that CHOP null mice have an equal level of apoptosis and an equal number of surviving dopamine neurons as compared to wildtype in the MPTP model.

We have found that two important markers of ER stress, the BiP chaperone protein, and the 422 bp splice variant of the transcription factor XBP-1, are not upregulated in the adult 6OHDA model, or the chronic or acute MPTP models. It is therefore possible that the upregulation of CHOP in these neurotoxin models is not mediated by ER stress, but rather another form of cellular stress, such as oxidative injury, which has been postulated to occur in both the 6OHDA and the MPTP models.

This conclusion is supported by data obtained in the current funding period, that homozygous null mutations in caspase-12, an important mediator of ER stress-induced apoptosis, do not protect in the 6OHDA model.

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Journal of Neurochemistry, 2005, **95**, 974–986 doi:10.1111/j.1471-4159.2005.03428.x

CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an *in vivo* neurotoxin model of parkinsonism

Robert M. Silva,* Vincent Ries,* Tinmarla Frances Oo,* Olga Yarygina,* Vernice Jackson-Lewis,* Elizabeth J. Ryu,§ Phoebe D. Lu,¶ Stefan J. Marciniak,¶ David Ron,¶ Serge Przedborski*,†,‡ Nikolai Kholodilov,* Lloyd A. Greene†,‡ and Robert E. Burke*,†

*Departments of *Neurology and †Pathology, ‡The Center for Neurobiology and Behavior, and §The Institute of Human Nutrition, The College of Physicians and Surgeons, Columbia University, New York, USA*

¶Departments of Medicine and Cell Biology, The Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, USA

Abstract

There is increasing evidence that neuron death in neurodegenerative diseases, such as Parkinson's disease, is due to the activation of programmed cell death. However, the upstream mediators of cell death remain largely unknown. One approach to the identification of upstream mediators is to perform gene expression analysis in disease models. Such analyses, performed in tissue culture models induced by neurotoxins, have identified up-regulation of CHOP/GADD153, a transcription factor implicated in apoptosis due to endoplasmic reticulum stress or oxidative injury. To evaluate the disease-related significance of these findings, we have examined the expression of CHOP/GADD153 in neurotoxin models of parkinsonism in living animals. Nuclear expression of CHOP protein is observed in developmental and adult models of dopamine neuron death induced by intrastriatal injection of 6-hydroxydopamine (6OHDA) and in models induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). CHOP is a mediator of neuron death in the adult 60HDA model because a null mutation results in a reduction in apoptosis. In the chronic MPTP model, however, while CHOP is robustly expressed, the null mutation does not protect from the loss of neurons. We conclude that the role of CHOP depends on the nature of the toxic stimulus. For 6OHDA, an oxidative metabolite of dopamine, it is a mediator of apoptotic death.

Keywords: apoptosis, endoplasmic reticulum stress, oxidative stress, Parkinson's disease, programmed cell death, substantia nigra.

J. Neurochem. (2005) **95,** 974–986.

There is an emerging consensus that programmed cell death (PCD) is likely to play a role in neuron death in neurodegenerative disease (Mattson 2000; Yuan and Yankner 2000). For Parkinson's disease (PD), this consensus is based on studies in animal models and human post-mortem material demonstrating either apoptotic morphology or immunohistochemical evidence for activation of caspases (reviewed in Vila and Przedborski 2003). One of the hallmarks of PCD is that in many contexts, it requires the transcription of genes that mediate cell death (Martin *et al*. 1988; Oppenheim *et al*. 1990). Therefore, a useful strategy to attempt to identify genes that mediate neuronal degeneration is to screen gene expression in models of disease. Such a strategy has been implemented for PD by performing serial analysis of gene

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Address correspondence and reprint requests to Robert E. Burke, Department of Neurology, Room 308, Black Building, Columbia University, 650 West 168th Street, New York, NY 10032, USA.
E-mail: rb43@columbia.edu

Abbreviations used: ABC, avidin-biotinylated-horseradish peroxidase complexes; ER, endoplasmic reticulum; MFB, medial forebrain bundle; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NRISH, non-radioactive *in situ* hybridization; 6OHDA, 6-hydroxydopamine; PB, phosphate buffer; PBS, phosphate-buffered saline; PCD, programmed cell death; PD, Parkinson's disease; PLD, post-lesion day; PND, post-natal day; SSC, saline sodium citrate; SN, substantia nigra; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

expression in PC12 cells, a catecholaminergic cell line (Greene and Tischler 1976), treated with 6-hydroxydopamine (6OHDA), a neurotoxin which is an oxidative metabolite of endogenous dopamine (Senoh and Witkop 1959; Kostrzewa and Jacobowitz 1974). Among the up-regulated transcripts identified by this analysis, and of particular potential relevance to neuronal death, was a striking induction of the transcription factor CHOP/GADD153 (Ryu *et al*. 2002). CHOP has been implicated as a mediator of apoptosis in the contexts of both endoplasmic reticulum (ER) stress (Matsumoto *et al*. 1996; Zinszner *et al*. 1998; Kawahara *et al*. 2001; Maytin *et al*. 2001; Gotoh *et al*. 2002; Oyadomari and Mori 2004) and oxidative stress (Guyton *et al*. 1996; Mengesdorf *et al*. 2002). In keeping with a possible role of either of these forms of cellular stress in mediating CHOP induction and neuron death, the analysis of gene expression also identified the induction of many other genes involved in ER and oxidative stress (Ryu *et al*. 2002, 2005).

A similar induction of CHOP was also observed by Holtz and O'Malley in a gene expression screen of neurotoxin models of parkinsonism (Holtz and O'Malley 2003). These investigators used Affymetrix gene arrays to screen dopaminergic MN9D cells following exposure to either 6OHDA or MPP^+, and noted that the most highly expressed transcript, for both neurotoxins, was that for CHOP (Holtz and O'Malley 2003).

These findings in gene expression screens performed *in vitro* are potentially relevant to human PD because the classes of transcripts induced, those related to oxidative stress and ER stress, relate to important current hypotheses for pathogenesis. The possibility that the oxidative metabolism of dopamine may be injurious to dopaminergic neurons is one of the longest-standing hypotheses (Fahn and Cohen 1992). More recently, ER stress has been postulated to play a role. An important genetic cause of PD is loss of function mutations in *parkin* (Ishikawa and Tsuji 1996; Kitada *et al*. 1998). These mutations have been implicated in abnormal protein processing because parkin is an E3 ubiquitin-ligase (Shimura *et al*. 2000) and, as such, it plays a role in targeting cellular proteins for destruction by the proteasome (Ciechanover 1998). One putative protein target of parkin, Pael-R, is a difficult-to-fold protein, and it has been postulated that its accumulation may result in dopaminergic neuron death due to ER stress (Imai *et al*. 2000, 2001).

The possible implications of these *in vitro* observations for the pathogenesis of PD depend on whether they generalize to the *in vivo* context. We have therefore investigated the expression of CHOP in several neurotoxin models of parkinsonism in living animals: substantia nigra (SN) dopamine neuron degeneration induced by intrastriatal injection of 6OHDA in both developing (Marti *et al*. 1997) and adult rodents (Sauer and Oertel 1994), and by both the acute (Heikkila *et al*. 1984) and chronic (Tatton and Kish 1997) systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). In addition, we have sought to determine whether CHOP plays a functional role as an essential mediator of dopamine neuron death by examining the vulnerability of homozygous CHOP null mice.

Materials and methods

Animals

For the study of postnatal rats, timed pregnant females were obtained from Charles River Laboratories (Wilmington, MA, USA). The date of delivery was defined as postnatal day (PND) 1. For adult mouse studies utilizing the 6OHDA and MPTP models, C57BL/6 mice were obtained from Charles River. CHOP null mice were produced by homologous recombination to replace all of the CHOP coding sequence (except for the final 34 C-terminal residues) with the coding sequence for β-galactosidase containing a nuclear localization signal. The neomycin selection cassette was then removed by Cre recombinase. There was no detectable CHOP protein in cells and tissues derived from these animals (Fig. 1). These mice were back-crossed into the C57BL/6 strain five times before breeding for experiments. The CHOP null mice were genotyped by PCR analysis of tail DNA using three-primer PCR analysis as previously described (Zinszner *et al*. 1998), with the modification that the primer to detect the mutant allele was based on the β-galactosidase sequence and produced a 300 bp product.

Animal models

The models used in this investigation are summarized in Table 1. The 6OHDA model in postnatal rats was performed as previously described (Marti *et al*. 1997). Briefly, rat pups at PND7 were pretreated with 25 mg/kg desmethylimipramine, anesthetized by hypothermia and placed prone on an ice pack. 6-OHDA hydro-

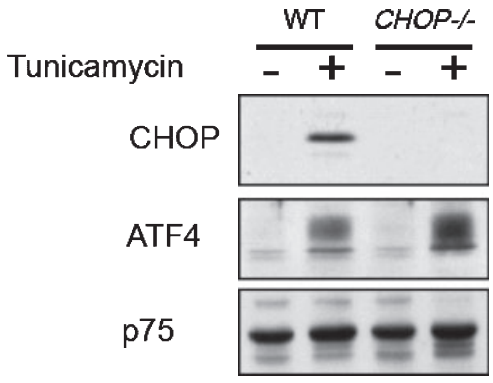

Fig. 1 Absence of CHOP protein expression in CHOP null mice. Immunoblot of nuclear extract of untreated and tunicamycin-treated (2 μg/mL, 6 h) wild-type and CHOP–/– cells blotted with antisera reactive with CHOP, ATF4 (a positive control) and p75, a ubiquitously-expressed nuclear protein that serves as a loading marker. No protein CHOP expression is observed in CHOP null cells after tunicamycin treatment. The antibody to ATF4 was raised against a full-length bacterially-expressed fusion protein and is characterized in Ron and Habener (1992). The p75 band was detected by an antiserum to *Drosophila* protein, described in Immanuel *et al.* (1995).

Table 1 Models used to assess the role of CHOP/GADD153 in apoptosis in SN dopamine neurons

Treatment	Species	Age	Route	Morphology of cell death
None (natural cell death)	Rat	Developmental	N.A.	Apoptosis
Axotomy	Rat	Developmental	N.A.	Apoptosis
6OHDA	Rat or Mouse	Developmental	Intrastriatal	Apoptosis
6OHDA	Mouse	Adult	Intrastriatal	Apoptotic and non-apoptotic
MPTP	Mouse	Adult	I.P., acute	Non-apoptotic
MPTP	Mouse	Adult	I.P., chronic	Apoptotic and non-apoptotic

Abbreviations: N.A., not applicable; I.P., intraperitoneal.

bromide (Regis, Morton Grove, IL, USA) was prepared at 15 μg (total weight)/1.0 μL in 0.9% NaCl/0.02% ascorbic acid, and infused by pump (Harvard Apparatus, Holliston, MA, USA) at a rate of 0.25 μL/min for 4 min (total dose 15 μg). Postnatal mice were injected in a similar fashion except that the solution was prepared at a concentration of 20 μg/μL and infused for 2 min, for a total dose of 10 μg. For experiments in postnatal mice, littermate wild-type and heterozygote animals were examined in comparison with nulls. Adult mice were infused with a concentration of 5 μg/μL at a rate of 0.5 μL/min for 8 min for a total dose of 20 μg. For experiments in adult mice, C57BL/6 adults were used as controls.

The medial forebrain bundle (MFB) axotomy model in postnatal rats was performed as previously described (El-Khodor and Burke 2002). Briefly, rat pups were anesthetized by hypothermia. Animals were positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA) to conform with the neonatal brain atlas of Heller *et al*. (1979). The MFB was transected by lowering a retractable wire knife (Kopf Instruments) through a skull burr hole 1.4 mm posterior and 2.5 mm lateral to bregma to a ventral position of 6.5 mm below bregma.

For the acute MPTP lesion model, mice received four i.p. injections of MPTP-HCl (20 mg/kg free base; Sigma, St Louis, MO, USA) dissolved in saline, 2 h apart in 1 day as previously described (Teismann *et al*. 2003). Control mice received saline only. MPTP handling and safety measures were in accordance with our published guidelines (Przedborski *et al*. 2001). For the chronic MPTP model, mice received one i.p. injection of MPTP-HCl per day (30 mg/kg per day of free base) for 5 consecutive days as described (Tatton and Kish 1997).

All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University.

Immunohistochemistry

For CHOP immunoperoxidase histochemistry, animals were perfused intracardially first with 0.9% NaCl and then with 4% paraformaldehyde and 0.1 M phosphate buffer (PB). The brains were then removed and post-fixed in the same fixative for 3 h. Each brain was then cryoprotected in 20% sucrose for 24 h. The brains were then rapidly frozen in isopentane on dry ice, and sections were cut in a cryostat at 30 μm. Sections were processed free-floating. After a phosphate-buffered saline (PBS) wash and treatment with PBS, 0.5% bovine serum albumen and 0.1% Triton X-100, sections were incubated with rabbit anti-CHOP at 1 : 500 for 48 h at 4°C. After a wash, sections were then incubated with biotinylated protein A (prepared in this laboratory) at 1 : 100 for 1 h at ambient room temperature. Sections were further incubated with avidin-biotinylated-horseradish peroxidase complexes (ABC; Vector Laboratories, Burlingame, CA, USA) at 1 : 600 for 1 h. After incubation with diaminobenzidine, sections were mounted onto subbed slides and counterstained with thionin. The primary antibody had been previously characterized and used for immunohistochemistry (Ron and Habener 1992; Zinszner *et al*. 1998). For immunofluorescence double-labeling for CHOP and tyrosine hydroxylase (TH), sections were collected into Tris-buffered saline (TBS) and then treated with TBS/0.2% Triton/2% goat serum/2% horse serum. They were then incubated in the same solution with anti-CHOP (1 : 500) and mouse anti-TH (1 : 40) (Chemicon, Temecula, CA, USA) for 48 h at 4°C. The sections were next treated with Texas red horse anti-mouse (Vector) at 1 : 75 and biotinylated goat anti-rabbit (Vector) at 1 : 75 for 1 h at ambient room temperature, followed by treatment with Fluor-avidin (Vector) at 1 : 100 for 1 h. Sections were then mounted onto gelatin-coated glass slides and coverslipped with Dako antifade medium (Carpinteria, CA, USA). The sections were examined by epifluorescence with a Nikon Eclipse 800 microscope.

For TH immunoperoxidase histochemistry, animals were perfused, as described above, and then post-fixed in the same fixative for 1 week. Each brain was cryoprotected in 20% sucrose for 24–48 h and then rapidly frozen. A complete set of serial sections through the SN was cut at 30 μm. Sections were saved individually in serial order at 4°C, and individual sections at regular intervals were then selected for TH immunostaining, in conformity with the fractionator method of sampling (Coggeshall and Lekan 1996) (see below). Sections were processed free-floating, as described above for CHOP. The primary antibody was a rabbit anti-TH (Calbiochem, La Jolla, CA, USA) at 1 : 1000. After treatment with biotinylated protein A and ABC, sections were mounted on subbed slides in serial order and thionin-counterstained.

Quantitative morphology

For the analysis of the time course of appearance of CHOP-positive nuclear profiles and apoptosis in the postnatal 6OHDA model in rats, counts were performed as previously described (Oo *et al*. 2003; Ganguly *et al*. 2004). CHOP-positive nuclear profiles were counted in identical fashion on the same sections.

The number of SN dopaminergic neurons in the lesion experiments with CHOP null and C57BL/6 control mice was

determined by stereological analysis. A complete set of TH-immunostained serial sections, sampled as every fourth section through the SN, was analyzed by a stereological method for each animal. Each analysis was performed under blinded conditions on coded slides. For each animal, the SN on each side of the brain was analyzed. For each section, the entire SN was identified as the region of interest. Using StereoInvestigator software (Micro Bright Field, Inc., Williston, VT, USA) a fractionator probe was established for each section. The number of TH-positive neurons in each counting frame was then determined by focusing down through the section, using a 100× objective under oil, as required by the optical dissector method (Coggeshall and Lekan 1996). Our criterion for counting an individual TH-positive neuron was the presence of its nucleus either within the counting frame, or touching the right or top frame lines (green) but not touching the left or bottom lines (red). The total number of TH-positive neurons for each SN on one side was then determined by the StereoInvestigator program. The total volume of the SN was also determined by the StereoInvestigator program for each brain on the basis of the sum of volumes derived from the area of each individual serial section and the tissue height represented by that section.

Northern analysis and non-radioactive *in situ* hybridization analysis (NRISH) of BiP

Rat BiP cDNA was subcloned into pCMS-EGFP (BD Biosciences, San Jose, CA, USA) as described (Ryu *et al*. 2002) and used for creation of an antisense RNA probe. Northern analysis was performed as previously described (El-Khodor *et al*. 2001). Briefly, RNA was isolated from microdissected SN using the Qiagen RNAeasy Mini kit (Valencia, CA, USA). The RNA concentration of each sample was determined by measuring absorption at 260 nm on a GenQuant spectro-photometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). A 20 μg aliquot of each RNA was electrophoresed in 1.4% agarose-formaldehyde gel and transferred onto an Immobilon (+) membrane (Millipore, Bedford, MA, USA). The hybridization was performed overnight at 68°C in Ultrahyb buffer from Ambion (Austin, TX, USA). The membrane was then exposed to phosphorimager cassettes, scanned and analyzed by Image Quant software (Molecular Dynamics, Indianapolis, IN, USA).

For NRISH, brains were rapidly removed from 6OHDA-injected adult mice at 48 h post-injection, and rapidly frozen in embedding medium on dry ice. Sections (14 μm) were thaw-mounted on glass slides (Superfrost Plus, Fisher, Hampton, NH, USA). For hybridization, sections were warmed on a slide warmer at 37°C for 20 min, and then fixed by immersion in 4% paraformaldehyde in 0.1 M PBS. After washing, sections were acetylated by treatment with acetic anhydride in triethanolamine. After another wash, sections were treated with a pre-hybridization solution, as previously described (Burke *et al*. 1994), for 2 h at ambient room temperature. Sections were then covered with hybridization solution and incubated overnight at 68°C in a humidified chamber. Hybridization solution contained the BiP riboprobe labeled with digoxigenin-UTP (1 μL/slide) (200–400 ng/mL), prepared as per the manufacturer's instructions (Roche Diagnostics, Penzberg, Germany). The size and integrity of labeled probe were confirmed by gel electrophoresis. The same probe used for northern analysis was used for the *in situ* hybridization. After a wash in 0.5× saline sodium citrate (SSC) for 10 min, followed by a wash in 0.2× SSC at 68°C for 30 min, sections were incubated with an anti-digoxigenin antibody (Roche) at 1 : 5000 overnight at 4°C. After additional washes, sections were incubated with a solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega Corporation, Madison, WI, USA) in a darkened humidified chamber overnight. Sections were then washed and coverslipped with Dako aqueous mounting medium.

RT-PCR/Southern blot analysis of the XBP-1 splice variant

To perform Southern analysis of the x-box binding protein-1 (XBP-1) splice variant, we first generated a DNA probe. We performed reverse transcription using RNA isolated from mouse kidney after treatment with tunicamycin. We then performed PCR of the 422 bp region of mouse XBP-1 containing the site of the unconventional splice, using primers based on nucleotide number 363 (Accession no. BC029197) (5′- CCTTGTGGTTGAGAACCAGG-3′) (forward) and nucleotide number 810 (5′-GAGGCTTGGTGTATACATGG-3′) (reverse). The band containing the spliced DNA fragment of XBP-1 was isolated from an agarose gel, subcloned in the pGEM-T vector (Promega) and sequenced. The DNA fragment containing the site of the XBP-1 unconventional splice was isolated from this clone using SalI and NcoI restriction enzymes (Promega). This fragment was then used to generate a ^{32}P-labeled DNA probe with the Rediprime II Kit, random prime labeling system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For XBP-1 splice variant Southern blot analysis, RNA was isolated from tissues using the Qiagen RNAeasy Mini Kit, as described above. First strand cDNA was then synthesized from isolated RNA by the RT system (Promega). PCR was performed individually with each cDNA sample using the above primers with Taq polymerase from Roche. A 10 μg aliquot of each DNA sample was electrophoresed in a 2% agarose gel. The DNA was then transferred onto a Hybond-N membrane (Amersham Pharmacia Biotech), hybridized with the XBP-1 DNA probe in Ultrahyb solution (Ambion) overnight at 42°C, washed as recommended, then exposed to phosphorimager cassettes, scanned and analyzed by Image Quant software (Molecular Dynamics).

Statistical analysis

The time course of appearance of apoptotic and CHOP-positive profiles in the postnatal 6OHDA model was analyzed by ANOVA with a Tukey post hoc analysis. Stereological determination of the number of SN dopaminergic neurons in the 6OHDA and MPTP lesion experiments was analyzed by ANOVA with a Tukey post hoc analysis. The number of apoptotic profiles in wild-type and CHOP null adult mice in the 6OHDA model was analyzed by the *t*-test. All statistical analyses were performed using SigmaStat software (SPSS Science, Chicago, IL, USA).

Results

CHOP protein expression is induced in a developmental neurotoxin model of parkinsonism

We initially performed *in vivo* experiments in a rat developmental model in which the intrastriatal injection of 6OHDA results in the induction of death in dopamine neurons of the SN, exclusively with the morphology of

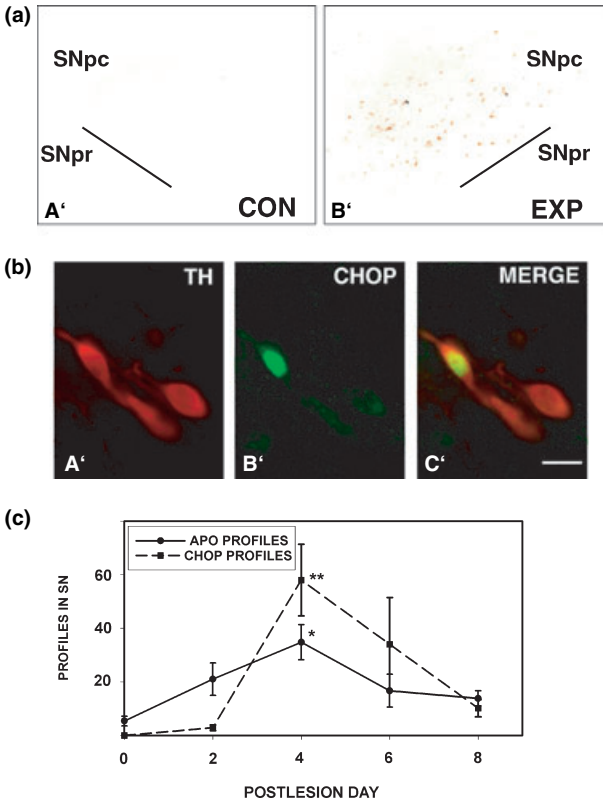

Fig. 2 Localization and time course of CHOP expression following developmental 6OHDA lesion in postnatal rats. (a) Low power photomicrographs at PLD6 of the substantia nigra contralateral (control: Con) and ipsilateral (experimental: Exp) to an intrastriatal injection of 6OHDA in a PND7 rat. CHOP protein expression is demonstrated by immunoperoxidase staining without a counterstain. CHOP-positive nuclei therefore appear as punctate brown profiles at this power. On the contralateral control side (a′), there is an absence of staining. On the ipsilateral experimental side, numerous CHOP-positive profiles are observed within the SNpc (b′). No positive profiles were observed in the SNpr or in the midbrain dorsal to the SNpc. (b) Double-immunofluorescence labeling for CHOP and TH in the SNpc at PLD4 following intrastriatal injection of 6OHDA in a PND7 rat. TH immunostaining is demonstrated by Texas Red (a′), CHOP by fluorescein (b′), and the merged image is shown in c′. CHOP immunostaining was predominantly nuclear. Following 6OHDA injection, CHOP staining was observed strictly within TH-positive, dopaminergic profiles of the SNpc. Note that CHOP-positive profiles appear normal morphologically; there is no apparent change in neuronal shape or proximal dendrites in comparison with adjacent, CHOP-negative, TH-positive neurons. Bar in c′ = 10 μm. (c) Time course for the appearance of apoptotic and CHOP-positive profiles in SN following intrastriatal injection of 6OHDA in PND7 rats. A total of 24 rats was studied: $n = 4$ at PLD0 and 2; $n = 5$ at PLD4 and 6; $n = 6$ at PLD8. CHOP-positive and apoptotic profiles were counted in the same sections from each animal, as described in Methods. The number of CHOP-positive profiles reached a peak at PLD4 (**$p < 0.02$ vs. PLD0, 2 and 8; ANOVA, Tukey post hoc). The number of apoptotic profiles also reached a peak at PLD4 (*$p < 0.05$ vs. PLD0 and 8; ANOVA, Tukey post hoc). However, the time of induction for the two types of profile differed at PLD2; for apoptotic profiles, the number at PLD2 was induced and not significantly different from the number at peak, whereas for CHOP profiles, there was no induction at PLD2. As discussed in the text, this difference may suggest that there are non-CHOP-dependent, as well as CHOP-dependent mechanisms of cell death in this model.

apoptosis (Marti *et al*. 1997). In this model, the unilateral intrastriatal injection of 6OHDA resulted in the unilateral induction of CHOP protein expression, demonstrated by immunohistochemistry (Fig. 2a). On the side of injection, CHOP expression was observed only in the SNpc, the exclusive site of neuron death in this model (Marti *et al*. 1997). CHOP expression was characterized at a cellular level by performing double-label immunofluorescence for CHOP and TH, to identify dopaminergic neurons of the SN. This analysis revealed that CHOP was expressed predominantly in the nucleus (Figs 2b,b′). To determine the cellular sites of CHOP expression within the SNpc, we examined 50 representative CHOP-positive nuclear profiles among six sections derived from two animals. This analysis showed that all CHOP-positive nuclei were within TH-positive, dopaminergic neurons of the SNpc. Thus, there was a precise correlation at the cellular level between the neuronal population that undergoes death in this model, and CHOP expression (Fig. 2b). All of the CHOP- and TH-positive neuronal profiles identified by the double-labeling procedure had a normal neuronal morphology: abundant cytoplasm, with a polygonal shape, and tapered proximal dendrites. We know from previous studies of this model that the vast majority of dopamine neurons die (Marti *et al*. 1997) and therefore, CHOP-positive profiles (all of which were TH-positive) are exceedingly likely to be destined to die. We therefore interpret the normal-appearing morphology to mean that if CHOP is to be implicated as a death mediator, it is expressed early in the death process, before any morphological change at the cellular level.

We investigated the time course of CHOP expression at the population level in this model. We recognize that since apoptosis occurs rapidly (Oppenheim 1991), and since at any given time of killing of the animal there will be a heterogeneous population of dying cells in varying stages of the death process, this population analysis will not resolve the cellular sequence of events. Nevertheless, it is informative to determine whether, at the population level, the appearance of CHOP-positive profiles correlates with the appearance of apoptotic profiles. CHOP expression at the population level in this model correlated at most times with the induction of apoptotic death (Fig. 2c). The occurrence of the peak number of CHOP-positive nuclear profiles corresponded precisely with the occurrence of the peak number of apoptotic profiles at postlesion day (PLD) 4. However, one exception to this correlation was that apoptosis was induced as early as PLD2, in the absence of any induction of CHOP, suggesting that an early component of apoptosis in this model is not associated with CHOP induction, as discussed further below.

Having demonstrated a co-localization between CHOP expression and the dopaminergic neuronal phenotype, and a temporal correlation between CHOP expression and apoptosis in the SN, we next examined the generality of the relationship in other developmental models in which apoptosis occurs. During the postnatal development of SN dopamine neurons, there is naturally-occurring cell death, exclusively with the morphology of apoptosis (Janec and Burke 1993; Oo and Burke 1997). Immunostaining for CHOP was performed on SN sections obtained from PND 14 rats (during the second phase of naturally-occurring cell death). We examined 36 SN sections among $n = 4$ rats and no instance of CHOP positivity was identified. Among these sections, 124 apoptotic profiles were identified, due to natural cell death (Fig. 3a). This naturally-occurring cell death can be augmented by an axotomy lesion of the medial forebrain bundle during the postnatal period (El-Khodor and Burke 2002). Examination of 18 SN sections from three PND6 rats at 24 h post-axotomy failed to reveal any CHOP-positive profiles (Fig. 3b). Among these sections, numerous apoptotic profiles were identified in SN, as described (El-Khodor and Burke 2002), and sections from 6OHDA-treated animals processed in parallel were positive for CHOP (Fig. 3c). Thus, we conclude that in the postnatal developmental period, CHOP protein expression is induced by the neurotoxin 6OHDA, but not by naturally-occurring cell death or a physical lesion that augments it.

CHOP protein expression in adult neurotoxin models of parkinsonism

To investigate the expression of CHOP in adult neurotoxin models, we exclusively studied mice to permit comparison between the 6OHDA model and the widely used MPTP mouse model of parkinsonism (Heikkila *et al.* 1984; Przedborski and Vila 2003). Adult mice injected into the striatum with 6OHDA demonstrated numerous CHOP-positive nuclei within neurons of the SNpc (Fig. 3d). For the study of MPTP effects on CHOP expression, we evaluated two dose regimens in common current use. Most widely used is an acute set of injections, 20 mg/kg for four doses, 2 h apart on a single day. This dosing regimen induces SN dopamine neuron death in the absence of apoptotic morphology (Jackson-Lewis *et al.* 1995). A second regimen utilizes a chronic set of injections, 30 mg/kg daily for 5 days (Tatton and Kish 1997), and results in neuron death with the morphological characteristics of apoptosis. In both of these

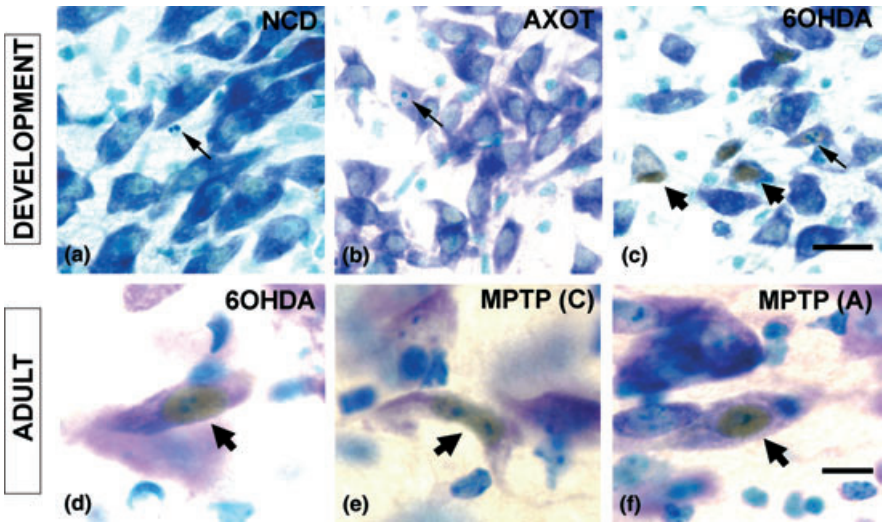

Fig. 3 CHOP is expressed in neurotoxin models of induced death in SN dopamine neurons. CHOP immunoperoxidase histochemistry was performed on free-floating sections, as described in Methods, with rabbit anti-CHOP (Zinszner *et al.* 1998) at 1 : 500 for 48 h, followed by thionin counterstain. (a) CHOP expression does not occur in SN during the apoptotic postnatal natural cell death event. A representative field showing a single apoptotic profile (arrow) in a PND14 rat is negative for CHOP immunostaining. (b) The naturally-occurring cell death event in SN can be augmented by postnatal axotomy of the dopaminergic axonal projection (El-Khodor and Burke 2002), as it is for many other developing neural projections (Oppenheim 1991). As for natural cell death, CHOP expression does not occur in this context, as shown by a representative field in a PND6 rat at 1 day post-lesion. An apoptotic profile is shown (arrow). (c) Unlike naturally-occurring cell death and axotomy, cell death induced by 6OHDA in PND7 rat results in the expression of CHOP in many neuronal profiles in the SNpc (broad arrowheads). In this model, CHOP-positive profiles rarely show basophilic apoptotic chromatin clumps (narrow arrow) (2% of instances). As discussed in the text, this rare association between CHOP expression and apoptotic nuclear morphology suggests that if CHOP is implicated in mediating death, it is likely to be an early participant, typically before morphological change. Bar = 20 μm for a, b and c. (d) A representative neuronal profile with a CHOP-positive nucleus (broad arrow) is shown at PLD6 following intrastriatal injection of 6OHDA in an adult mouse. (e, f) CHOP nuclear staining is also observed in SNpc neurons following MPTP injection in adult mice by either the chronic (C) or acute (A) regimens. Bar = 10 μm for d, e, f.

MPTP models, numerous CHOP-positive neuronal profiles were identified within the SN (Figs 3e and f). In all of these adult contexts, positive nuclear CHOP expression was identified in neurons which otherwise appeared normal, suggesting, as previously discussed, that if CHOP is to be implicated as a death mediator in these models, then it is expressed prior to degenerative morphological changes. We conclude from these studies that CHOP is generally expressed in the SNpc in neurotoxin models of parkinsonism.

CHOP mediates neuron death in the adult 6OHDA model

Having demonstrated close relationships between CHOP expression and the death of SN dopamine neurons in these neurotoxin models, we next sought to determine whether CHOP plays a critical functional role in mediating this death, as it has been shown to do in non-neuronal models of cell death due to ER stress (Zinszner *et al*. 1998) and oxidative stress. For this assessment, we compared the sensitivity of homozygous CHOP null mice with wild-type controls in their degree of sensitivity to neurotoxin-induced neuron death. In the postnatal 6OHDA model, we found that there was no difference between homozygous CHOP nulls and either heterozygous mice or wild-type controls in the degree of apoptosis among SN dopaminergic neurons induced by intrastriatal 6OHDA (Fig. 4). However, we recognized that in this model, death is known to be mediated not only by the direct effect of the neurotoxin but also, in the developmental period, by an 'axotomy' effect due to destruction of dopaminergic terminals during a period of target dependence (Marti *et al*. 1997). Since we had shown directly that axotomy does not induce CHOP expression, we considered the possibility that this admixture of death mechanisms may obscure a role played by CHOP in death due to the neurotoxin. Such a possibility was also suggested by the time course analysis in Fig. 2(c), which showed an early apoptotic component in the absence of CHOP induction. We therefore examined the sensitivity of adult CHOP null mice to intrastriatal injection of 6OHDA, as adult dopamine neurons do not have target dependence (Kelly and Burke 1996).

In adult mice, there was a clear protective effect of the homozygous CHOP null mutation (Fig. 5). CHOP null animals demonstrated a 65% reduction in the number of apoptotic profiles in the SNpc at the sixth post-lesion day. To determine whether this reduction in the magnitude of neuron death resulted in a lasting protection from the neurotoxin, we examined the number of surviving TH-positive neurons in the SN at 28 days post-lesion. This analysis revealed that the null mutation did provide a substantial, lasting protective effect; there was a 79% increase in the number of surviving TH-positive neurons in comparison with wild-type controls (control: 857 ± 131; null: 1531 ± 173 neurons per SN) (Fig. 5b). Nevertheless, the absolute magnitude of the protective effect in the nulls, expressed as 31% survival,

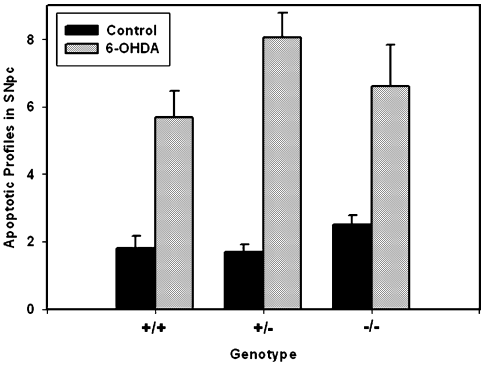

Fig. 4 The CHOP null mutation does not protect from induction of apoptosis in the developmental 6OHDA model. In total, 20 PND6 mice (wild-type $n = 5$; heterozygous $n = 10$; null $n = 5$) received a unilateral intrastriatal 6OHDA injection and were killed at PLD4 for the determination of apoptotic profiles within the TH-immunostained substantia nigra, as described in Methods. In all three genotypes, there was a robust induction of apoptosis, as previously described for rats (Marti *et al.* 1997) (ANOVA $p < 0.001$ for the 6OHDA effect). There were, however, no differences among the genotypes for this effect.

while significantly greater than that in the wild-type (19%, $p < 0.02$), was considerably less than anticipated based on a 65% suppression of apoptotic death in the acute period. In addition, at 28 days post-lesion, there was no evidence for sparing of dopaminergic innervation of the striatum in the nulls. In the nulls, there was a 28.0 ± 3.2 sparing of the optical density of TH-positive fibers within the striatum, as there was in wild-type controls (28.3 ± 3.6).

CHOP mediates a cellular response to injury, but not neuron death, in the chronic MPTP model

Given that CHOP expression is induced in both the acute and chronic MPTP models, we sought to determine whether it plays a role as a death mediator, as it does in the adult 6OHDA model. Since the role of CHOP as a death mediator has previously been identified in non-neuronal cells in the context of apoptosis (Zinszner *et al*. 1998), we examined its role in the chronic MPTP model in which apoptosis has been identified (Tatton and Kish 1997). Based on our results in the adult 6OHDA model demonstrating a disparity between the ability of the CHOP null mutation to protect from death in the acute period following the lesion as compared with the chronic period, we conducted separate assessments of both of these post-lesion periods. We found that the CHOP null mutation provided a protective effect in the acute (PLD4) period following the chronic administration of MPTP. The CHOP null animals demonstrated only a non-significant trend for a decrease in the number of TH-positive SN neurons at this time, whereas wild-type controls demonstrated a 65% decrease (Figs 6a and b). However, this difference could not be attributed to a difference in the magnitude of apoptotic death between the two genotypes. While there was

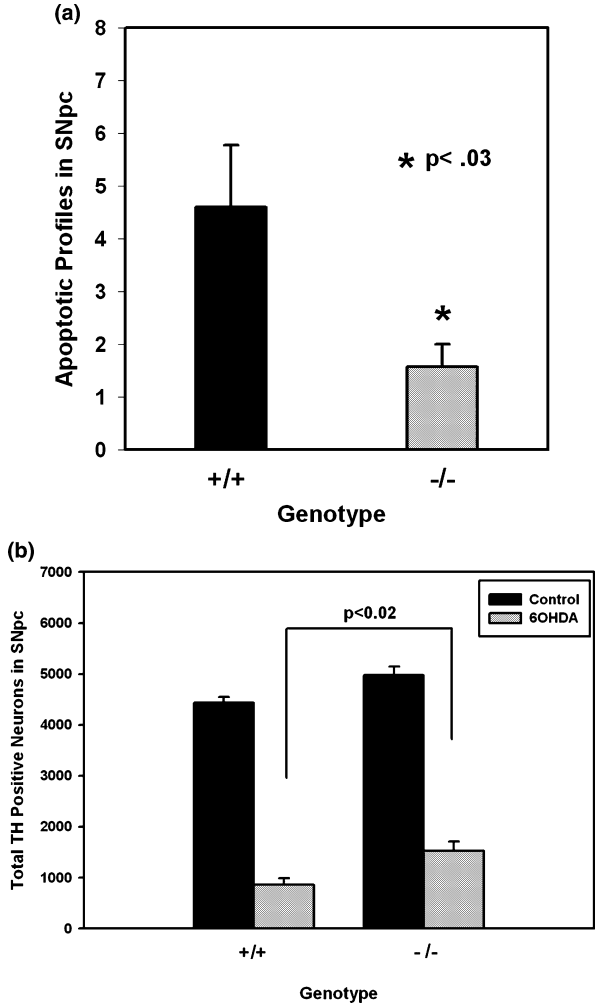

Fig. 5 The CHOP null mutation protects from apoptotic cell death in the adult 6OHDA model. (a) Wild-type ($n = 5$) and CHOP homozygous null ($n = 6$) adult mice were injected into the striatum with 6OHDA. They were killed 6 days later for TH immunostaining of the SN and counting of apoptotic profiles within the SNpc. The CHOP null animals demonstrated a 65% reduction in the level of apoptosis ($p < 0.03$, *t*-test). (b) Wild-type ($n = 7$) and CHOP null ($n = 8$) adult mice were injected with 6OHDA and killed 28 days later for TH immunostaining of serial sections for stereologic determination of the number of surviving dopaminergic neurons. In both genotypes, the 6OHDA injection led to a significant reduction in the number of SN dopamine neurons ($p < 0.001$, ANOVA; Tukey post-hoc). In the CHOP null animals, there was a 79% increase in the number of surviving neurons ($p < 0.02$, Tukey post hoc). Nevertheless, the absolute magnitude of preservation of neurons (31%) was less than anticipated, based on a much greater level of suppression of death in the acute phase.

a trend towards fewer apoptotic profiles in these sections among the CHOP null mice (2.7 ± 0.8 profiles/SN), this did not achieve significance in comparison with the wild-type (5.2 ± 1.1, $p > 0.1$, Tukey post hoc). We therefore attribute the marked difference in number of TH-positive neurons between the two genotypes to the well described suppression of TH phenotype following MPTP treatment (Jackson-Lewis *et al*. 1995). In keeping with this interpretation, in the chronic setting at 21 days post-lesion, there was only a 36% decrease in TH neuron number following MPTP in the wild-type animals. This apparent increase in the number of TH-positive neurons between the acute and chronic lesion periods has previously been shown to be due to a recovery of phenotype (Jackson-Lewis *et al*. 1995). In the chronic period, in the MPTP-treated mice, unlike the 6OHDA-treated mice, there was no protective effect of the null mutation on the number of surviving TH-positive neurons (Fig. 6c). This difference between the two models is in keeping with the lack of an effect of the null mutation on the magnitude of cell death in the acute period of the MPTP model, whereas there was a pronounced effect in the 6OHDA model. As would be expected from the lack of a protective effect of the null mutation on TH-positive neuron number, there was also no protective effect on striatal TH-positive fiber density (data not shown). We therefore conclude that in the chronic MPTP model, CHOP appears primarily to play a role in the loss of phenotype response that accompanies cellular injury, rather than in cell death, as it does in the 6OHDA model.

CHOP induction in neurotoxin models is not accompanied by changes in BiP mRNA expression, or the appearance of the XBP-1 splice variant

The induction of CHOP alone cannot be taken as compelling evidence for the occurrence of the ER stress response because CHOP can be induced by other cell stressors, such as oxidative stress, arsenite exposure and amino acid limitation (Bruhat *et al*. 1997; Jousse *et al*. 1999; Entingh *et al*. 2001; Mengesdorf *et al*. 2002). Therefore, to determine whether the induction of CHOP observed in these models was indicative of the broader ER stress response, we examined the mRNA expression of an ER-resident chaperone, BiP (also know as Grp78) (Gething 1999; Kaufman 1999). Induction of BiP mRNA has previously been shown to occur *in vitro* in conjunction with CHOP induction upon exposure of neuronal cells to 6OHDA (Ryu *et al*. 2002; Holtz and O'Malley 2003). In addition, we assessed nigral tissue by PCR for the presence of a splice variant of the transcription factor x-box binding protein-1 (XBP-1) (Yoshida *et al*. 2001; Calfon *et al*. 2002), a specific marker for the unfolded protein response.

Northern analysis of SN tissue from mice treated according to the chronic MPTP regimen on the last day of injection (PLD0) or 2 days after the final injection failed to demonstrate any change in BiP mRNA in comparison with saline-treated controls (not shown). To conduct an analysis of BiP mRNA expression at the SNpc regional and cellular levels in the adult 6OHDA model, we performed NRISH. As

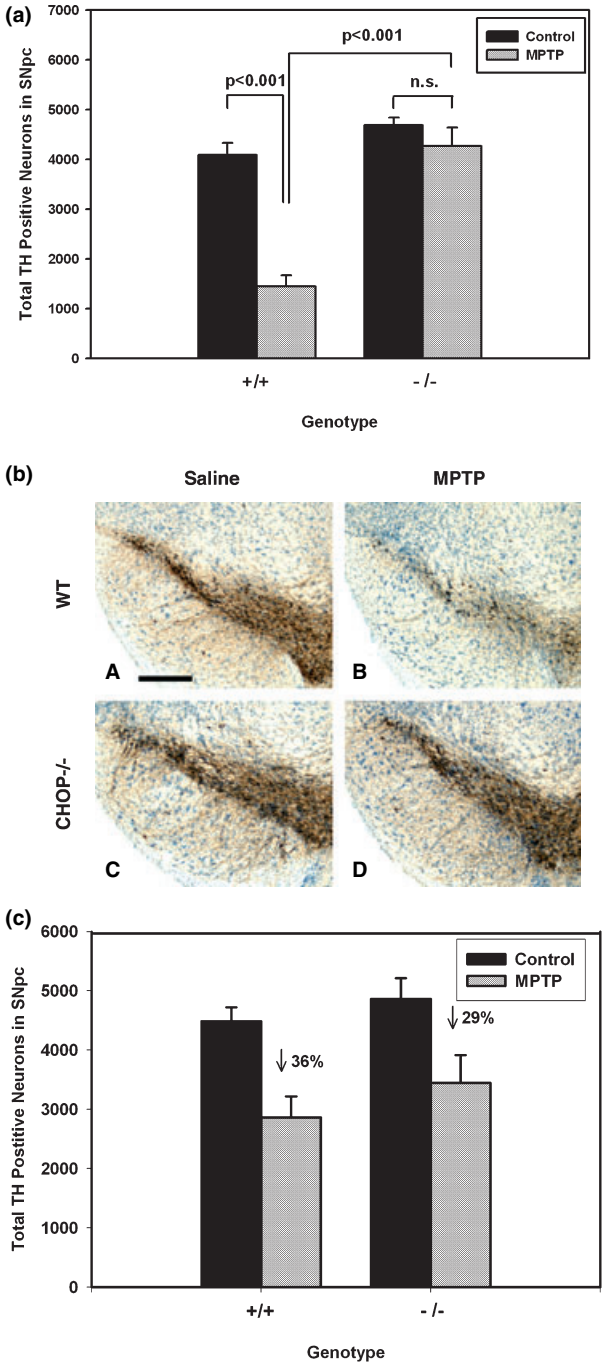

Fig. 6 The CHOP null mutation provides early protection from loss of phenotype, but not from neuron death, in the chronic MPTP model. (a) Wild-type and CHOP null adult mice were injected with saline or MPTP (30 mg/kg/day) for 5 days ($n = 4$ each group except wild-type saline, $n = 3$) and killed 4 days after the last dose for immunostaining and stereologic determination of TH-positive neuron number. Remarkably, there was minimal apparent effect in the CHOP nulls treated with MPTP. The wild-type mice showed a 65% decrease in number of TH-positive profiles. This difference could not be attributed to a change in the magnitude of apoptosis, as discussed in the text. (b) Representative low power photomicrographs demonstrating the resistance of SN dopamine neurons in CHOP null mice to the early effect (4 days post-lesion) of MPTP in the chronic injection model. These sections are derived from mice studied by stereologic analysis of TH-positive neuron number, shown in (a). Bar = 300 μm. (c) Wild-type and CHOP null mice were injected with saline or MPTP ($n = 4$–5 each group) and killed 21 days following the final injection for TH immunostaining and stereology. At this late post-lesion day, when the acute suppression of phenotype has recovered, it is apparent that there has been only a 36% loss of SN dopamine neurons in wild-type mice. While there was a trend for a reduction in the number of neurons lost in the CHOP null mice (29% loss), this did not achieve significance ($p > 0.5$, Tukey post-hoc).

previously reported by others for normal rat (Little *et al*. 1996), we observed widespread constitutive expression of BiP mRNA in brain (not shown). However, we did not observe any induction in SNpc, at the regional or cellular level, following unilateral intrastriatal 6OHDA injection at PLD2. A similar analysis of MPTP-treated mice failed to show any difference in BiP mRNA expression in SNpc in comparison with saline-treated controls (not shown).

Southern analysis of PCR reaction products for the XBP-1 unspliced and spliced variants was performed with the inclusion of a positive control derived from renal tissue of tunicamycin-treated mice, in which the ER stress response has previously been demonstrated (Zinszner *et al*. 1998). This analysis was performed for SN tissues derived from 6OHDA-treated mice at 1 and 3 days post-lesion, and for tissues derived from mice treated with MPTP according to both the acute and chronic regimens. In no instance was the XBP-1 splice variant identified in SN tissues, in spite of its clear presence in tunicamycin-treatment renal tissue. We conclude that in spite of the induction of CHOP protein in these models, there is no additional biochemical evidence of an unfolded protein response using these methods at the tissue level.

Discussion

The hypothesis that PCD plays a role in neural degeneration in PD rests principally on two forms of evidence. First, in rodent neurotoxin models, there is histological and biochemical evidence for activation of PCD mediators, such as the caspases, and functional evidence from genetic and pharmacological studies (reviewed in Vila and Przedborski 2003). Second, while traditional morphological assessments of human PD post-mortem brains for apoptosis have been controversial, there has been growing evidence for activation of caspases (Hartmann *et al*. 2000, 2001; Viswanath *et al*. 2001). While this evidence validates PCD as a target for the development of neuroprotective therapeutics, much remains unknown, particularly about upstream mediators that would make attractive therapeutic targets (Yuan and Yankner 2000).

The identification of CHOP as a markedly up-regulated transcript following the treatment of catecholaminergic cell lines with dopaminergic neurotoxins (Ryu *et al*. 2002; Holtz and O'Malley 2003) and with rotenone, a mitochondrial Complex 1 inhibitor (Ryu *et al*. 2002), is of particular interest because as a transcription factor, it would be likely to play an upstream regulatory role. In keeping with that possibility, a gene activated by CHOP, DOC6, is homologous to gelsolin, a mediator of cytoskeletal collapse during apoptosis (Wang *et al*. 1998). CHOP is also of particular interest in relation to PD because it has been implicated as an apoptotic mediator in the setting of oxidative stress (Guyton *et al*. 1996; Mengesdorf *et al*. 2002), which has been long postulated to play a role in PD (reviewed in Fahn and Cohen 1992), and ER stress (Matsumoto *et al*. 1996; Zinszner *et al*. 1998; Kawahara *et al*. 2001; Maytin *et al*. 2001; Gotoh *et al*. 2002; Oyadomari and Mori 2004), which has likewise recently been implicated in this disease (Imai *et al*. 2000, 2001).

We have determined that CHOP is expressed in neurotoxin animal models of parkinsonism. In a developmental model of apoptosis induced in dopamine neurons of the SN by the intrastriatal injection of 6OHDA (Marti *et al*. 1997), there was robust induction of CHOP protein expression exclusively within the SNpc. At a cellular level, CHOP expression was nuclear, as expected for a transcription factor, and exclusively within dopaminergic neurons. CHOP expression was also observed in neurotoxin models in the adult setting following intrastriatal 6OHDA, and either acute or chronic systemic MPTP exposure. In these adult models, as in the developmental 6OHDA model, CHOP expression was strictly within the SNpc at a regional level, and within the nucleus of otherwise normal-appearing neurons at a cellular level. CHOP expression, however, is not a universal feature of apoptosis in dopamine neurons; in the developmental setting, it is observed neither during naturally-occurring cell death (Janec and Burke 1993; Oo and Burke 1997), nor with augmentation of this death by axotomy (El-Khodor and Burke 2002). On the basis of classic neurotrophic theory (Clarke 1985), the naturally-occurring cell death event and its augmentation by axotomy would be considered to be regulated by the availability of neurotrophic support. Our observations that CHOP is not induced in these conditions, but it is by neurotoxic insults, are comparable with the *in vitro* observations of Ryu *et al*. (2002), who noted that CHOP is induced by neurotoxins, but not by neurotrophic withdrawal.

The principal finding of these investigations was that adult CHOP null mice were resistant to apoptotic death in SN dopamine neurons induced by the intrastriatal injection of 6OHDA. We considered the possibilities that this reduction may be due to a change in the time course of apoptosis, or to the rate of clearance of apoptotic profiles in the null mice, rather than an actual reduction in the eventual magnitude of the death event. We therefore assessed the final surviving number of SN DA neurons at PLD28 and found that they were increased, indicating that the null mutation did in fact reduce the magnitude of death. We therefore conclude that CHOP is an important functional mediator of apoptosis in the 6OHDA model. Given that CHOP is highly expressed prior to any morphologic change in dopamine neurons destined to die in this model, we postulate that CHOP is likely to be an early mediator in the death process. Although the CHOP null mutation was protective in this model, the degree of preservation of SN dopamine neurons in absolute terms, 31%, was less than anticipated based on a 65% suppression of apoptotic death in the early post-lesion period. This discrepancy suggests that some of the death which ultimately occurs in the CHOP nulls is delayed. There are two possible explanations for this delay. First, death mediators other than CHOP may eventually come into play (Ryu *et al*. 2005) and bring about the loss of the majority of dopamine neurons. Second, in these non-temporally-regulated nulls, compensatory changes may have taken place to provide alternate death pathways. These two possibilities are not mutually exclusive.

In view of the ability of the CHOP null mutation to provide neuroprotection in the adult 6OHDA model, the question arises as to why it did not also provide protection in the postnatal model, in which CHOP expression is clearly induced. Our interpretation of this difference rests on our previous demonstrations that during the first two postnatal weeks, SN dopamine neurons are dependent on interactions with their target, the striatum, as envisioned by classic neurotrophic theory (Clarke 1985), whereas in adults they are not (Macaya *et al*. 1994; Kelly and Burke 1996; Stefanis and Burke 1996). Therefore, during this postnatal period, the death of SN dopamine neurons following the destruction of their nerve terminals with 6OHDA is likely to be mediated by an 'axotomy' effect as well as a direct neurotoxic effect. This interpretation is supported not only by the aforementioned studies of the developmental time course of striatal target dependence, but also by our demonstrations that the postnatal 6OHDA model is characterized by two cellular patterns of caspase activation: a perinuclear pattern, as observed in naturally-occurring cell death (Jeon *et al*. 1999; El-Khodor and Burke 2002; Oo *et al*. 2002), and a cytoplasmic pattern, observed in direct neurotoxic injury (Jeon *et al*. 1999; Oo *et al*. 2002). Given this likelihood of an axotomy effect in the postnatal 6OHDA model, and based on our demonstration herein that CHOP is not expressed following developmental axotomy, we would anticipate that a functional role for CHOP would be difficult to discern in the postnatal 6OHDA lesion.

MPP^+, the toxic metabolite of MPTP, induced CHOP expression in *in vitro* models (Ryu *et al*. 2002; Holtz and O'Malley 2003). MPTP treatment *in vivo* likewise induced the expression of CHOP protein, but in the chronic MPTP

model, unlike the 6OHDA model, the CHOP null mutation did not significantly diminish the level of apoptosis or increase the number of surviving neurons. The null mutation did, however, prevent the loss of TH immunoreactivity in the period early after the MPTP injections. We interpret this relative preservation of TH immunoreactivity in the absence of protection from cell death to be attributable to protection from the loss of phenotype, which is well documented in this (Jackson-Lewis *et al*. 1995) and other neuronal injury models (Wooten *et al*. 1978). We conclude that while CHOP plays a role in regulating cellular phenotype in the MPTP model, it is not likely to play a role as an important death mediator. This difference in the role of CHOP between the 6OHDA and MPTP models in living animals is consistent with the observations made *in vitro* by Holtz and O'Malley (2003). Following treatment with 6OHDA, they observed a greater induction of CHOP and a more general induction of other ER stress markers than with MPTP treatment.

To determine whether the CHOP induction observed in these neurotoxin models was specifically due to ER stress, we assayed mRNA expression of the ER-resident chaperone BiP (Gething 1999; Kaufman 1999) and the splice variant of XBP-1 (Yoshida *et al*. 2001; Calfon *et al*. 2002). In none of the models was there a change observed in BiP mRNA expression or the appearance of the XBP-1 splice variant. These results were not unexpected for the MPTP model in view of *in vitro* results that showed no induction of BiP or XBP-1 by MPP^+ (Holtz and O'Malley 2003). However, the results were unexpected for the 6OHDA model as both prior *in vitro* studies had demonstrated clear evidence for a full ER stress response induced by 6OHDA (Ryu *et al*. 2002; Holtz and O'Malley 2003). There are two principal interpretations of these negative results. First, it is possible that CHOP induction in the 6OHDA model in living animals is not part of a full ER stress response, the *in vitro* results notwithstanding. It is well established that 6OHDA produces oxidative stress (Heikkila and Cohen 1973; Cohen and Heikkila 1974). It is therefore possible that its induction of CHOP in living animals is mediated principally by cellular oxidative stress (Guyton *et al*. 1996; Mengesdorf *et al*. 2002). Alternatively, it is possible that the studies of BiP and the XBP-1 splice variant that were performed at the tissue level lacked the sensitivity to detect changes, which, for CHOP, were detected at the cellular level by immunohistochemistry. Thus, our inability to detect other markers for ER stress in the 6OHDA model does not permit us to definitively conclude that it is not present.

We conclude that these investigations performed in living animals are largely supportive of the *in vitro* results suggesting the possibility of a role for CHOP in the neurodegeneration associated with PD. We find, as predicted from these gene expression screens, that CHOP is expressed in diverse neurotoxin models of dopamine neuron death. These observations support the validity of the *in vitro* screens for genes of potential relevance to disease. In addition, we find that CHOP can play a role as a mediator of cell death, depending on the context; in the 6OHDA model, CHOP is a necessary death mediator. The context specificity of CHOP is an important feature, because it suggests that it may be possible in designing neuroprotection strategies to target disease-related death pathways without interfering with other apoptotic pathways that may be important for survival of the organism.

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Research Review

Mixed Lineage Kinase–c-jun N-Terminal Kinase Signaling Pathway: A New Therapeutic Target in Parkinson's Disease

Robert M. Silva, PhD,[1] Chia-Yi Kuan, PhD,[2] Pasko Rakic, MD, PhD[3] and Robert E. Burke, MD[1,4]*

[1]*Department of Neurology, The College of Physicians and Surgeons, Columbia University, New York, New York, USA*
[2]*Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation, Cincinnati Ohio, USA*
[3]*Department of Neurobiology, Yale University School of Medicine, New Haven, Connecticut, USA*
[4]*Department of Pathology, The College of Physicians and Surgeons, Columbia University, New York, New York, USA*

Abstract: There is growing evidence that the molecular pathways of programmed cell death play a role in neurodegenerative disease, including Parkinson's disease, so there has been increased interest in them as therapeutic targets for the development of neuroprotective strategies. One pathway of cell death that has attracted particular attention is the mixed lineage kinase (MLK) –c-jun N-terminal kinase (JNK) signaling cascade, which leads to the phosphorylation and activation of the transcription factor c-jun. There is much evidence, from in vitro and in vivo studies, that this cascade can mediate cell death. In addition, there is evidence that it is operative upstream in the death process. It is possible that abrogation of this pathway may forestall death before irreversible cellular injury. One class of compounds that has shown promise for their ability to block cell death by inhibiting this cascade are the inhibitors of the MLKs, which are upstream in the activation of c-jun. One of these compounds, CEP1347, is now in a Phase II/III clinical trial for neuroprotection in PD. Whether this trial is successful or not, this signaling cascade is likely to be a focus of future therapeutic development. This review, therefore, outlines the principles of signaling within this kinase pathway, and the evidence for its role in cell death. We review the evidence that inhibition of the MLKs can prevent dopamine neuron cell death and the degeneration of their axons. These studies suggest important future directions for the development of therapies that will target this important cell death pathway. © 2005 Movement Disorder Society

Key words: apoptosis; programmed cell death; neurodegeneration; neuroprotection

The concept of programmed cell death arose largely from studies in developmental biology, which demonstrated that, during normal development, many cells are normally and predictably eliminated during morphogenesis and that such processes are highly conserved evolutionarily. Within the neurosciences, it had long been recognized that, during development, many neurons are normally lost due to cell death,[1,2] and many of the early authoritative reviews of the subject speculated that these processes may be relevant to adult-onset neurodegenerative disorders.[3,4] Approximately 10 years ago, there was a tremendous growth of interest in programmed cell death as a mechanism for human neurological disease.[5] This growth was probably fueled by several factors, primarily a rapid growth in our understanding of the molecular basis of these processes[6]; but also perhaps by the realization that these processes could re-emerge in adult animals.[7,8] In relation to dopamine neurons, in particular, interest in programmed cell death was supported by the demonstrations that they, like most neuronal populations, undergo natural cell death during development[9,10] and that they undergo apoptosis in neurotoxin models of Parkinson's disease (PD).[11,12]

There followed a period of frustration and controversy in the study of programmed cell death in neurodegenera-

*Correspondence to: Dr. Robert E. Burke, Department of Neurology, Room 308, Black Building, Columbia University, 650 West 168th Street, New York, NY 10032. E-mail: rb43@columbia.edu

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tive diseases, based on very mixed results of efforts to identify the characteristic morphology of apoptosis in human postmortem material.[13] In PD, whereas some investigators identified apoptosis,[14,15] others did not,[16,17] and even today there is not a consensus that apoptosis can be identified in the PD brain. There are many possible reasons for these mixed results. Apoptosis is a short-lived process, and it is likely to be exceedingly difficult to identify in chronic neurological diseases in which neuron death occurs gradually over years. In addition, apoptosis is only one of the known morphologies of programmed cell death[18] and its absence in tissue does not exclude a possible role for programmed cell death mechanisms. Ultrastructural analysis is required to identify these alternate morphologies, and it is exceedingly difficult to achieve a high-quality analysis in postmortem material. Thus, controversies and mixed results from studies of human postmortem material are not unexpected.

Despite these persistent controversies over studies of human material, there is a growing consensus that the processes of programmed cell death are exceedingly likely to play a role in neurodegeneration. This consensus is based on numerous studies in experimental animal models of human neurodegenerative diseases, which have revealed two types of evidence supportive of a role for programmed cell death. The first is that, with the development of a more detailed understanding of the molecular pathways, it has been possible to develop antibodies to activated proteins, such as the caspases, which mediate proteolysis of intracellular proteins, and to detect their presence, sometimes even in the absence of classic apoptotic morphology. Second, it has been possible to show in many of these models that abrogation of cell death pathways, by either pharmacological or genetic means, the latter by either transgenesis or gene delivery approaches, can forestall cell death. These developments for many neurodegenerative disorders have been thoroughly reviewed[19–21] and, therefore, will not be reviewed in detail here. It will suffice to say here that the consensus about the probable role of programmed cell death pathways in neurodegenerative disorders, irrespective of their diverse proximate causes, has been strong enough to impel much effort among academic investigators and the pharmaceutical industry to develop neuroprotective therapeutic approaches based on antagonism of critical death mediators. There especially has been an effort to antagonize death mediators upstream in cell death pathways, to abort the process before irreversible cellular injury.

This review will focus on the upstream mediators c-jun and its kinase (c-jun N-terminal kinase, or JNK), for which much evidence suggests a role in programmed cell death in neurons. We believe that it is now worthwhile for clinicians as well as basic neuroscientists to have a detailed knowledge of the role of these mediators in neuronal death. There is now under way a clinical trial to assess the ability of an inhibitor of c-jun activation, CEP1347, to provide neuroprotection in PD (The PRECEPT Trial, administered by the Parkinson Study Group). To the best of our knowledge, this is the first example of a compound that was developed primarily for its ability to block programmed cell death, in both tissue culture and animal models, to be subjected to a Phase II/III trial for neuroprotection in any neurodegenerative disease. Whether this particular compound is successful or not, we believe that it is likely that the JNK/c-jun signaling cascade will remain a therapeutic target and will be the focus of future trials. The likelihood of relevance of this pathway to neuron death in PD in addition is supported by the recent direct demonstration of abnormal, nuclear localization of c-jun in the dopaminergic neurons of PD brains.[22] Thus, we will herein review the molecular basis of c-jun regulation and the evidence of its role in neuron death, both generally and specifically within dopamine neurons.

MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAYS

All eukaryotic cells possess multiple mitogen-activated protein kinase (MAPK) pathways that are activated by a wide range of stimuli, including hormones and growth factors, inflammatory cytokines, and diverse environmental stresses, such as osmotic shock, ischemia, and ionizing radiation.[23] These pathways, in turn, have a variety of downstream effects, including the regulation of gene transcription, the cell cycle, cellular differentiation and cell death. All MAPK signaling pathways are organized in a three-tier signaling structure (Fig. 1) in which the MAPKs (in the first tier) are activated by phosphorylation of Tyr and Thr residues within a conserved Thr-X-Tyr motif. This phosphorylation is catalyzed by upstream kinases (the second tier), the MAPK kinases (MAPKKs), also known as the MAPK/extracellular signal-regulated kinases (ERKs) -kinases (MEKs or MKKs).[23,24] These kinases are, in turn, regulated by Ser/Thr phosphorylation, which is catalyzed by any of several MAPK-kinase-kinase (MAPKKK) families. Regulation of MAPKKKs is achieved by membrane recruitment, oligomerization, and phosphorylation. In some cellular contexts, this is mediated by GTPases of the Ras superfamily, such as Cdc42 and Rac proteins. A fundamental organizational principal of the MAPK pathways is that their complexity is managed in part by scaffold

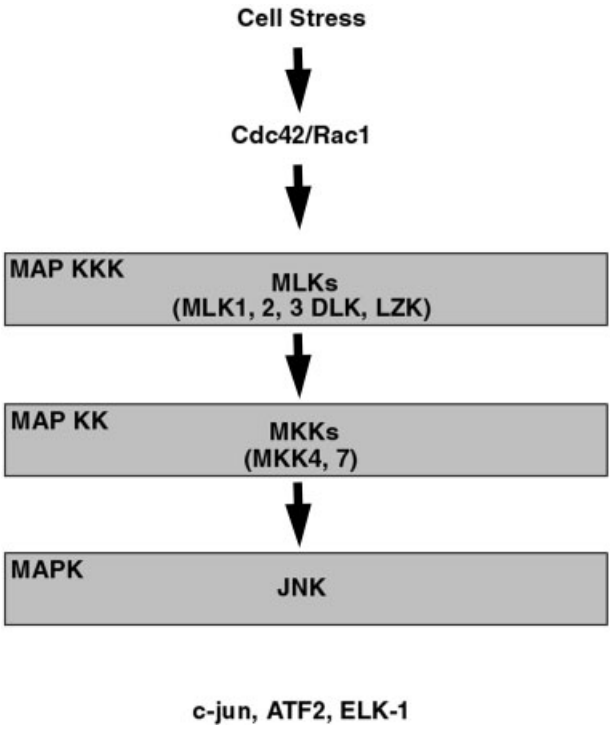

FIG. 1. The mitogen-activated protein kinase (MAPK) signaling pathways. MAPK signaling pathways are organized in a three tier structure. The c-jun N-terminal kinase (JNK), which mediates phosphorylation and activation of c-jun, is a MAPK in the first tier. Also in this group of kinases are the p38 MAPKs and the extracellular signal-regulated kinases (ERKs, not shown). The MAPKs are activated by phosphorylation of Tyr and Thr residues by the MAPK kinases in the second tier (see Table 1 for alternative names for members of the MAPK-kinases (MAPKKs). In this tier, MKK4 and MKK7, in particular, mediate activation of JNK. Upstream to the MAPKKs, in the third tier, several families of kinases have been reported to activate JNKs (see the text). Among these kinases, the mixed lineage kinases (MLKs) have been implicated in neuron death.

proteins that are capable of binding, sequestering, and fostering specific interactions among select components. This organization permits specific types of stimuli to produce unique and coordinated MAPK signaling responses.[23]

In mammalian cells, three different groups of MAPK have been identified: the JNKs (also known as the stress-activated protein kinases [SAPKs]), the p38 MAPKs, and the ERKs.[23] The JNKs clearly have been implicated in mediation of programmed cell death in neurons and will be the focus of this review. JNK (SAPK) was first identified in 1990 as a rat hepatic kinase for microtubule-associated protein 2, which was activated after cycloheximide injection.[25] Separate, parallel studies identified 46- and 55-kDa protein kinases capable of phosphorylating serine residues 63 and 73 in the N-terminal region of c-jun, thereby potentiating its transactivation function.[26] By molecular cloning SAPK and JNK were shown to be identical in 1994.[27,28] There are three genes encoding JNK protein kinases. The *Jnk1* and *Jnk2* genes are widely expressed, whereas *Jnk3* is expressed only in brain, heart, and testis.[24] Alternate splicing of the gene transcripts results in further molecular diversity. A splice site within the Jnk1 and 2 transcripts results in two splice forms; a second alternate splice for all JNK transcripts occurs at the C-terminus of the protein resulting in the 46- and 55-kDa protein isoforms. Thus, 10 isoforms have been identified. JNK has numerous substrates, as discussed below, but it is important to note in relation to c-jun in particular, one of its first identified substrates, that JNK remains the dominant kinase for c-jun in vivo; immunodepletion of JNKs from cell extracts removes all stress and cytokine-activated c-jun phosphorylation activity.[23]

Upstream activation of the JNKs is mediated primarily be two MAPKKs, MKK4 (also know as SAPK/ERK kinase-1, or SEK1; Table 1) and MKK7. Gene disruption of MKK4 and 7 eliminates JNK activation, indicating that they are major activators of JNK in vivo.[29] These two kinases act synergistically to phosphorylate and activate the JNKs. MKK4 preferentially targets JNK Tyr-185, whereas MKK7 preferentially phosphorylates Thr-183. In vitro, each kinase alone will active JNKs approximately 5- to 10-fold, but when added together, they activate them approximately 100-fold.[23] In addition, they appear to mediate JNK activation by different stimuli; MKK7 is activated primarily by cytokines (such as tumor necrosis factor [TNF] and interleukin-1 [IL-1]), whereas MKK4 is primarily activated by environmental stress.[24]

Upstream to the MAPKKs, several families of MAPKKKs have been reported to activate JNKs, including the MEK kinases (MEKKs 1–4; MEK is an alternate name for the MKKs; see Table 1), apoptosis signal-regulating kinase-1 (ASK1), transforming growth factor β-activated kinase1 (TAK1), tumor progression locus-2 (TPL-2), and the mixed lineage kinases (MLKs; see Kyriakis and Avruch[23] and Davis.[24] for complete reviews). The MLKs have been clearly implicated in programmed cell death in

TABLE 1. *MAPKK Nomenclature*

NAME	ALTERNATE NAMES
MEK1	MAPKK1, MKK1
MEK2	MAPKK2, MKK2
SEK1	MKK4, JNK kinase (JNKK)-1, MEK4, SAPK-kinase (SKK)-1
MKK7	JNKK2, MEK7, SKK4
MKK3	MEK3, SKK2
MKK6	MEK6, SKK3

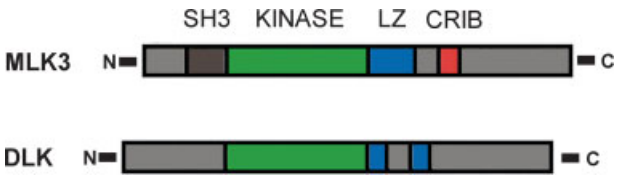

FIG. 2. The protein domain structures of mixed lineage kinase-3 (MLK3) and dual-leucine-zipper-bearing kinase (DLK). MLK3 and DLK provide representative examples of the domain structures of the MLKs. Common to all MLKs is the kinase domain (green), which mediates phosphorylation, and the leucine zipper domain (blue), which mediates protein–protein dimerization. Exemplified here by MLK3, the MLKs 1–4 contain an N-terminal Src-homology-3 (SH3) domain (brown), which may be involved in autoregulatory mechanisms. MLKs 1–4 also contain a Cdc42/rac-interactive binding (CRIB) motif (red) that is believed to mediate activation by Cdc42.

neurons, based on studies with the MLK inhibitor CEP1347[30] and dominate-negative genetic approaches,[31] so we will limit this review to this family of JNK activators.

In general, protein kinases contain 11 conserved subdomains. Of these, subdomains I to VII of the MLKs resemble serine/threonine kinases, whereas subdomains VIII to XI more closely resemble tyrosine kinases. Thus, when the MLK genes were initially cloned, they were termed "mixed lineage kinases." However, biochemical studies have shown that the MLKs serve as serine/threonine kinases. In recent years, three subfamilies of MLKs have been identified, containing seven different kinases.[32] Based on an analysis of domain arrangements and sequence similarities, the three families are the MLKs (containing MLKs 1–4); the dual-leucine-zipper-bearing kinases (DLKs, containing DLK and leucine-zipper kinases); and the zipper sterile-α-motif kinases (ZAK, containing ZAK alone). In rat substantia nigra (SN), mRNA for DLK is far more abundant than that for MLKs 1–3 or LZK,[33] but in human SN, the relative abundance is unknown. The domain structure for the MLK families can be exemplified by that of MLK3 and DLK (Fig. 2). MLKs 1–4 have an amino-terminal Src-homology-3 (SH3) domain, which apparently serves to autoinhibit the kinase activity.[32] Moving toward the C-terminal, this domain is followed by the kinase domain, containing the catalytic site for phosphorylation. There then follows a leucine zipper region, important for protein–protein interactions. It has been shown for DLK that the leucine zipper is required for self-association, phosphorylation, activation, and stimulation of the JNK pathway.[32] An MLK3 mutant lacking the zipper fails to autophosphorylate and activate JNK.[32] In MLKs 1–4, following the leucine zipper toward the C-terminal, there is a Cdc42/Rac-interactive binding motif (CRIB), which mediates interaction with these Rho family GTPases. MLK3, for example, is able to bind to activated forms of Cdc42 and Rac; when Cdc42 and MLK3 are coexpressed in cells, there is an increase in MLK3 activity and potentiation of JNK activation. All of the known members of the MLK family, when transfected into cells, act as MAPKKKs to activate JNK, and they do so by activation of MAPKKs such as MKK4 and MKK7.[23,32] While the MLKs are principally known for their ability to activate the JNKs,[23] some have been shown also to activate p38.[32]

In the context of JNK activation by cytokines, such as members of the TNF family, the MAPKKKs are activated by the binding of the cytokine ligand to cell surface receptors of the TNF receptor superfamily.[23,24] Binding induces oligomerization with signaling proteins, the TNF receptor (TNFR) -associated factor (TRAF) proteins either directly, as in the case of TNFR2, or indirectly through a TNFR-associated death domain protein (TRADD), as is the case for TNFR1. The TRAFs, in turn, can activate JNK by means of MAPKKKs.[23] One of the TRAFs, TRAF2, has also been shown to mediate the activation of JNK in the setting of endoplasmic reticulum stress.[34]

As stated, JNK was originally identified based on its ability to phosphorylate c-jun and thereby enhance its ability to transactivate other genes.[26] This ability to regulate gene transactivation remains its principal recognized role.[24] C-jun dimerizes with itself and other transcription factors (such as c-Fos and ATF) to constitute activator protein-1 (AP-1) transcription factors, which regulate the expression of several stress-responsive genes. N-terminal phosphorylation of c-jun increases its stability.[35] JNK also phosphorylates and activates other AP-1 proteins, including JunB, JunD, and ATF2. In addition, another transcription factor, ElK1, is a target of JNK phosphorylation; it is involved in induction of the c-fos gene. Thus, the role of JNK in mediating programmed cell death is likely to be, at least in part, through its role in regulating gene transcription, and this role is likely to be mediated through its ability to phosphorylate c-jun. This conclusion is supported by the observation that mutations of c-jun, substituting alanines for its serine 63 and 73 phosphorylation sites, lead to increased resistance to apoptosis in neurons,[36] as discussed further below.

However, JNK is unlikely to mediate apoptosis only through its effects on gene transcription, because, for example, inhibitors of transcription do not completely block ultraviolet (UV) light-induced apoptosis in fibroblasts, which is dependent on JNK.[37] There is increasing evidence that JNK is able to directly phosphorylate and regulate pro- and antiapoptotic activity of members of

the Bcl-2 family. Several studies have demonstrated that JNK can phosphorylate and diminish the antiapoptotic activity of both Bcl-2[38,39] and Bcl-X_L.[40] The latter study demonstrated that induction of apoptosis by irradiation is associated with translocation of JNK to mitochondria and binding to Bcl-X_L. In addition, JNK is capable of phosphorylating the proapoptotic protein BAD at serine 128 and potentiating its proapoptotic effect.[41] Similarly, JNK has been demonstrated to phosphorylate the proapoptotic proteins Bim and Bmf, thereby causing their release from sequestration by dynein motor complexes, with translocation to mitochondria, followed by release of mitochondrial death mediators.[42] Thus, JNK is likely to act by both transcriptional and nontranscriptional mechanisms to mediate death.

As stated, a general principal in the organization of the MAPK signaling cascades is that protein interactions are orchestrated in part by scaffolding proteins, which are able to bind specific protein components and foster interactions among them, thus permitting specific stimuli to produce unique signaling responses. This principal can be illustrated for JNK signaling by two such scaffolding proteins: JNK-interacting protein-1 (JIP1)[43] and POSH (plenty of SH3s).[44]

JIP1 was first identified as a specific JNK-interacting protein; it does not interact with the p38s or the ERKs.[43] As predicted for a scaffolding protein, it is able to bind multiple components involved in JNK activation: among the MAPKKKs, it interacts with MLK3 and DLK; among the MAPKKs, it interacts with MKK7. Coexpression of JIP1 with MLK3 or MKK7 enhances the ability of these kinases to activate JNK. JIP1 illustrates the role of scaffolding proteins to regulate the specificity of MAPK signaling, because JIP1 null animals show deficits in the activation of JNK due to excitotoxic stress or anoxia, but their neurons do not show deficits in activation due to UV radiation.[45] Expression of the JNK binding domain (JBD) alone (amino acids 127-281) apparently has dominate-negative effects, as it blocks nerve growth factor (NGF) -withdrawal–induced apoptosis,[43] and it has been used in vivo to demonstrate the role of JNK signaling in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity (see below).[46]

POSH was first identified as a Rac-interacting protein by the yeast two-hybrid method, and the interaction was shown to be GTP-dependent.[44] POSH was shown to induce JNK activation and apoptosis in non-neuronal cells.[44] Xu and coworkers demonstrated that POSH expression induces JNK phosphorylation and apoptosis in neural cells.[47] Furthermore, it is capable of direct interaction with the MLKs, MKKs 4 and 7, and the JNKs, and, as would be predicted, it acts upstream to the MLKs, the MKKs, and c-jun to induce cell death.[47] Thus, POSH is another example of a scaffold protein mediating cell death by the MAPK signaling cascade.

MAPK SIGNALING IN NEURONAL CELL DEATH IN VITRO

Over the past decade, a large body of in vitro evidence has emerged suggesting that c-jun plays an important role in the mediation of neuronal programmed cell death. One of the principal in vitro models for the study of cell death in neurons has been the use of sympathetic neurons isolated from rat superior cervical ganglia (SCG) subjected to the withdrawal of NGF. These neurons undergo a form of cell death that displays the morphological and biochemical features of apoptosis, including nuclear chromatin condensation, DNA fragmentation, attenuated rates of cellular transcription and translation, and diminished energy metabolism.[48–50] Early studies demonstrated up-regulation of c-jun protein[51] and mRNA[52] in sympathetic neurons after NGF withdrawal. Microinjection of a dominant-negative form of c-jun, which lacked the transcriptional activation domain, protected SCG neurons from NGF-withdrawal–induced cell death, whereas overexpression of wild-type c-jun protein resulted in significant induction of apoptosis even in the presence of NGF.[51] Similarly, microinjection of neutralizing antibodies for c-jun protein significantly reduced neuronal death after NGF withdrawal.[52] Identical treatment using neutralizing antibodies directed against other members of the AP-1 family, such as JunB and JunD, failed to demonstrate a protective effect. These studies clearly illustrated that c-jun function plays a significant role in the death of sympathetic neurons after trophic factor withdrawal. Similar results have also been reported in cerebellar granule cells after survival signal withdrawal,[53] and in differentiated PC-12 cells after NGF withdrawal.[54]

Activation of the c-jun pathway upon induction of programmed cell death has also been identified in tissue culture in cells that more closely resemble the dopaminergic phenotype. Holtz and O'Malley have investigated MN9D cells, which display many properties of living dopaminergic neurons, including the synthesis and storage of dopamine,[55] and primary mesencephalic cultures.[56] They identified up-regulation of both c-jun mRNA and c-jun phosphorylation in MN9D cells, and increased phosphorylation in primary cultures, after treatment with 6-hydroxydopamine (6-OHDA). Smaller, more transient changes were observed in MN9D cells after MPP+ treatment.

As the principal kinase for c-jun, JNK has also been implicated in programmed cell death in tissue culture

models. Withdrawal of NGF from PC12 cells leads to sustained activation of JNK.[57] In SCG cells, withdrawal of NGF induces increased JNK activity, serine 63 phosphorylation of c-jun, and transcriptional activation of the c-jun promoter.[58] Prevention of c-jun phosphorylation protects PC-12 cells from apoptosis after NGF withdrawal.[59]

Upstream regulators of JNK activity, such as members of the MLK family of MAPKKKs have also been shown to modulate c-jun activation and apoptosis. Overexpression of MLKs induced apoptotic cell death in PC12 cells[31] and SCG neurons.[60] On the other hand, expression of dominant-negative MLKs blocked NGF-withdrawal–induced apoptosis.[31,60] These findings support consideration of upstream regulators of c-jun activation as valid therapeutic targets for the treatment of neurodegenerative disorders.

The possibility of targeting the MLKs in particular has been supported by studies with the MLK inhibitor CEP1347. CEP1347 is a derivative of the indolocarbazole alkaloid K-252a, a natural product isolated from *Nocardiopsis* bacteria, which previously had been shown to promote cell survival in vitro.[61] K-252a, a serine/threonine kinase inhibitor,[62] promoted neurite outgrowth in human SH-SY5Y neuroblastoma cells,[63] as well as survival of chick dorsal root and ciliary ganglion neurons in culture.[64] In addition to its survival-promoting profile, K-252a was also found to protect neurons against injury induced by free radicals, β-amyloid–induced toxicity, and glucose-deprivation–induced death.[65,66] The survival and neuroprotective properties of K-252a led to the development of analogs that lacked the undesirable protein kinase C and trkA kinase inhibitory activities but retained the ability to enhance neuronal survival. Specifically, analogs were evaluated for their ability to enhance choline acetyltransferase (ChAT) activity in embryonic rat spinal cord and basal forebrain tissue cultures.[67] This analysis revealed that the 3,9-disubstituted K-252a (ethylthio)methyl derivative CEP1347 displayed a more-effective profile in both the spinal cord and basal forebrain ChAT assays, compared to K-252a. The significance of this finding is underscored by earlier studies demonstrating that ChAT up-regulation is observed in neuronal cultures treated with NGF or insulin-like growth factor and is associated with increased survival in motoneurons in vitro.[68–70]

After these initial discoveries, a series of experiments confirmed the neuroprotective effects of CEP1347 using an array of cellular insults. Maroney and colleagues[71] demonstrated that, after trophic factor withdrawal, rat motoneuron cultures treated with CEP1347 displayed nearly complete survival after 72 hours, whereas cell viability in untreated control cultures decreased by 65%. Moreover, an examination of the morphology of these motoneuron cultures revealed that, in contrast to CEP1347-treated cultures, which continued to display normal neuronal morphology for up to 5 days, untreated controls displayed a clearly apoptotic morphology, including cellular fragmentation, neurite retraction, and chromatin condensation after withdrawal of trophic factor. Survival in this study was shown to correlate with the inhibition of JNK1 activity. These findings were confirmed and extended in a subsequent study demonstrating that pretreatment with CEP1347 prevented NGF-withdrawal–, UV-irradiation–, and oxidative-stress–induced death in neuronally differentiated PC-12 cells and rat sympathetic neurons.[72] That these cellular insults induce death by means of three distinct pathways suggested that the mechanism of CEP1347 involved a shared molecular component, most likely, the activation of the JNK pathway.[73]

The role of the JNK cascade in CEP1347-mediated neuroprotection was further elucidated by findings demonstrating that CEP1347 inhibited both JNK activation and cell death induced by members of the MLK family in vitro.[30] These findings suggested that the protective effect of CEP1347 was a result of the inhibition of the MLKs, which lie upstream of JNK-mediated c-jun phosphorylation. A recent study[74] expanded these findings in relation to MPP+ toxicity by demonstrating that treatment with CEP-1347 or a dominant-negative MLK3 adenoviral construct inhibited MPP+-induced cell death as well as JNK signaling in neuronally differentiated human neuroblastoma SH-SY5Y cells. These experiments further suggested that CEP1347 mediated neuroprotection as a result of inhibition of MLK3. These findings are potentially relevant to PD, because differentiated SH-SY5Y cells share many characteristics of living dopaminergic neurons, including dopamine synthesis, expression of dopamine receptors, and uptake and sequestration of dopamine consistent with the expression of the dopamine transporter.[74–76]

MAPK SIGNALING IN NEURONAL CELL DEATH IN VIVO

Initial studies of c-jun expression in the central nervous system of living animals in models of injury were difficult to interpret in relation to cell death, because early studies in peripheral systems had shown that expression could be up-regulated by regenerative processes.[77] Such was also the case in some contexts of central injury; in a fimbria–fornix axotomy model, for example, in which death of medial basal forebrain neurons does not occur, there is a sustained increase in c-jun mRNA

and protein expression.[78] Therefore, in the earliest studies of c-jun expression at the regional level in injury models accompanied by neuron death, it was quite difficult in the diverse neuronal populations under study to specifically attribute cell death to c-jun expression. Nevertheless, early studies in a variety of ischemia models noted close associations in time and regional location between c-jun mRNA or protein expression and neuron death.[79–81] One particular study, by Dragunow and coworkers,[82] noted in a neonatal hypoxia–ischemia model a delayed expression of c-jun in neurons undergoing a delayed neuronal death as opposed to early necrotic death, and suggested that the former may be a form of programmed cell death.

The earliest studies specifically within the SN in models of death induced by 6-OHDA[83] and by axotomy[84] noted substantial and sustained increases in c-jun expression, but these changes were interpreted largely in relation to a possible role in regenerative responses. In the 6-OHDA model, however, the maximal expression of c-jun, at 4–8 days postlesion,[83] is when other investigators subsequently showed that cell death is maximal.[85]

With subsequent increased awareness of apoptosis as a distinct morphology of programmed cell death,[86] and the ability to detect it by nuclear staining, it became clear that c-jun expression could be correlated at the cellular level with this form of cell death in living animals. This finding was true in the context of natural cell death in the peripheral[87] and central[88] nervous systems, and in models of induced natural cell death.[89] Similarly, in the SN, close correlations could be made between c-jun expression and markers of apoptosis. Herdegen and colleagues[90] demonstrated in the adult axotomy model a close regional and temporal association between prolonged c-jun expression and terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) labeling for apoptosis.[91] Oo and associates[92] demonstrated in a postnatal model of apoptosis in the SNpc, induced by early target deprivation, that c-jun and JNK expression could be correlated at a cellular level with apoptotic morphology. Thus, these morphological studies of apoptotic cell death suggested a clear correlation with c-jun expression.

The first principal evidence for a functional role for JNK/c-jun signaling in cell death in living animals derived from studies in JNK null animals. Yang and coinvestigators reasoned that, because the JNK3 isoform is selectively expressed in the nervous system, it may play a role in neuronal death. They showed that JNK3 null mice are indeed resistant to kainic acid-induced seizures and associated hippocampal neuron apoptosis.[93] These animals also demonstrated diminished levels of c-jun phosphorylation and AP-1 transcriptional activity. Whereas this study demonstrated a clear role for this JNK isoform in mediating cell death, it remained an open question whether c-jun itself was the relevant substrate for this effect; other JNK3 substrates, such as ATF2, EIK-1, or unknown substrates, were also possible. To address the precise role of c-jun, Behrens and colleagues created mice by homologous recombination in which the endogenous c-jun gene was replaced by an altered gene in which the serines at positions 63 and 73 were replaced by alanines, which cannot be phosphorylated.[36] Mice homozygous for this mutant, nonphosphorylatable form of c-jun were also resistant to seizures and hippocampal neuron apoptosis induced by kainate. Thus, the phosphorylation of c-jun by JNK appears to be responsible for apoptosis in this model.

A functional role for c-jun in mediating death specifically within dopamine neurons has been supported by studies using viral vector gene transfer approaches. Crocker and coworkers[94] have demonstrated in an axotomy model that adenovirus-mediated expression of a c-jun dominant-negative construct not only prevents the loss of dopamine neurons in the SN but also the loss of dopaminergic fibers in the striatum. Because this latter protection occurs in the presence of axotomy, it presumably is independent of nuclear transcriptional events, but the precise mechanism is unknown. A functional role for JNK/c-jun signaling in dopamine neuron death is also supported by the demonstration that gene transfer of the JNK binding domain of JIP-1 (which inhibits JNK activation) protects dopamine neurons from chronic MPTP toxicity.[46] Again, this approach not only prevented the loss of SN dopamine neurons, but also their striatal terminals, as assessed by catecholamine levels. The functional significance of the latter was demonstrated by behavioral benefit.

In view of this evidence that phosphorylation of c-jun plays a role in the mediation of cell death in dopamine neurons and given that JNK is the dominant kinase for c-jun,[23] it would be predicted that JNK isoforms also play a role in the death of these neurons. Hunot and coinvestigators have shown in a model of acute MPTP toxicity that both JNK2 and JNK3 homozygous null animals are resistant; each genotype shows only approximately a 50% reduction of SN dopaminergic neurons, much less than controls.[22] JNK1 null animals were not protected. Compound mutant JNK2 and 3 homozygous nulls were even more protected; they showed only a 15% loss of neurons. Thus, both JNK2 and JNK3 play a role in cell death in this model. The compound null mutation also protected dopaminergic fibers in the striatum. These investigators postulated that increased transcriptional ac-

tivity mediated by JNK phosphorylation of c-jun may mediate cell death, and they found, by Gene Chip microarray analysis, that the immune mediator cyclooxgenase-2 is up-regulated. JNK was shown to be necessary for this up-regulation, as it was abolished in the compound JNK mutants. Thus, JNK may ultimately act, at least in part, in the acute MPTP model by up-regulation of cycloogenase-2, which has been implicated as a death mediator in this model.[95]

These results should not be construed, however, as direct evidence for a role for JNK as a mediator of apoptotic death within dopamine neurons. The principal reason is that apoptosis does not occur in the acute MPTP model,[96] whereas it does in the chronic MPTP model.[12] Another important difference between the two models is that a major inflammatory component occurs in the acute model, whereas it is much less in the chronic model.[97] Therefore, although it is clear that JNK plays an important role in dopamine neuron death in the presence of inflammation in the acute model, it remains to be determined if it is necessary for cell-autonomous apoptotic death within dopamine neurons and if it plays a role in the death of these neurons in other contexts. These issues are of course important for therapeutic implications for PD. It is important in this regard to note that the functional effects of JNK activation depend a great deal on the cellular context. For example, dramatic increases in JNK activity can occur in physiological contexts, such as exposure to novel environments, in which cell death does not occur.[98] In fact, during embryonic development, the JNK1 and 2 isoforms appear to play a role in suppressing apoptosis; in compound mutant mice homozygous null for both isoforms, there is an increased amount of apoptosis in the developing forebrain.[99]

These studies, based on genetic techniques using either gene transfer or transgenesis in mice, indicating a functional role for MAPK signaling in the mediation of neuron death in living animals, have received much support from pharmacological studies using the specific MLK inhibitors CEP1347, described earlier, and its analogue CEP11004.[100] As summarized above, there is much evidence that CEP1347 can abrogate programmed cell death in a variety of tissue culture models using many different types of cellular insult. Efficacy of CEP1347 to forestall programmed cell death has also been observed in diverse living animal models. Glicksman and colleagues demonstrated that application of CEP1347 prevented 40% of the natural cell death in spinal motor neurons that normally occurs in embryonic chicks.[101] They also showed that CEP1347 had the ability to diminish postnatal natural cell death in spinal motor neurons in rats. This compound has also been demonstrated to forestall pathological cell death in injury models. In an excitotoxic injury model, induced by intracerebral injection of ibotenate, CEP1347 protected basal forebrain cholinergic neurons.[102] In a model of apoptosis induced in auditory hair cells by noise trauma, CEP1347 diminished the loss of cells and protected hearing.[103]

These MLK inhibitors have also been shown to be protective in animal models of parkinsonism. In a single dose model of MPTP toxicity, Saporito and coinvestigators demonstrated that CEP1347 attenuated the loss of dopaminergic terminal markers and cell bodies in SN, demonstrated by immunostaining.[104] In the MPTP single-dose model, there is increased phosphorylation of JNK and the upstream kinase MKK4 and these increases are attenuated by CEP1347.[105] A similar ability to inhibit the phosphorylation of MKK4 and prevent the loss of dopaminergic terminals in the single-dose MPTP model was also demonstrated for CEP11004.[100] In the acute MPTP model, Teismann and associates[95] demonstrated that CEP11004 inhibited phosphorylation of c-jun, diminished the loss of tyrosine hydroxylase (TH) -positive neurons, and prevented increases in cyclooxygenase-2. Because these models did not directly examine the occurrence of cell death and because, as discussed above, apoptosis does not occur in the acute MPTP model, it was important to determine whether MLK inhibition could directly forestall apoptotic death within SN dopamine neurons. In a model characterized by the exclusive induction of apoptosis in these neurons by intrastriatal injection of 6-OHDA in postnatal rats, CEP11004 diminished the number of dopaminergic apoptotic profiles.[33] This death is mediated, at least in part, by the intrinsic, mitochondrial pathway, because it is associated with an induction of the activated, cleaved form of caspase 9. CEP11004 acts upstream to this point, because it diminished the number of caspase 9–positive profiles in proportion to overall protection from cell death.[33] A notable result of this study was an almost complete protection of striatal TH-positive fibers; especially remarkable considering that the toxin was injected directly into striatum. Overall, these studies demonstrate a clear neuroprotective effect of these MLK inhibitors in a variety of living animal models of parkinsonism.

It is important to note that, when evaluating the promise of agents that appear to be neuroprotective in preclinical studies, all the animal models currently used, particularly the neurotoxin models commonly used, have limitations. These neurotoxin models, although they reliably induce dopamine neuron death, remain of uncertain relevance to human PD. In addition, compounds are assessed for neuroprotective properties by administration

before or soon after administration of the toxin. In this respect, the compounds are not being tested in a context that is as demanding as the clinical context, when 70 to 80% or more of dopamine terminals have been lost by the time of diagnosis. Clearly, more clinically relevant and more demanding models are needed.

MAPK SIGNALING IN HUMAN POSTMORTEM BRAIN IN NEURODEGENERATIVE DISEASE

To date, evidence for the involvement of c-jun in human neurodegenerative disease has been limited. Nevertheless, several studies have implicated this pathway in the pathophysiology of human neurodegenerative disease, particularly Alzheimer's disease (AD). Anderson and coworkers first reported increased intensity of c-jun immunostaining in the hippocampus and entorhinal cortex of AD brains, in comparison to controls.[106] The c-jun immunoreactivity was colocalized with staining for paired helical filaments. These investigators subsequently also observed a relationship between c-jun immunostaining and TUNEL labeling for DNA strand breaks.[107] MacGibbon and colleagues[108] also found some evidence for increased immunostaining for c-jun in AD postmortem hippocampus, but it was less striking than that reported by Anderson and associates. However, by using a quantitative approach, Marcus and coworkers also identified an increased number of c-jun–positive profiles in AD hippocampus, compared to age-matched controls.[109] Such differences were not observed in a control region, the cerebellum.

Evidence of a role for MAPK signaling in PD brain has been mixed. Recently, Hunot and colleagues[22] have reported evidence of c-jun activation in postmortem tissue recovered from idiopathic Parkinson's disease patients. A quantitative analysis of c-jun–positive, pigmented neurons in SNpc revealed that a greater proportion showed a nuclear localization among the PD patients. Ferrer and colleagues identified phosphorylated JNK immunoreactivity rarely in the cytoplasm of some neurons, in the vicinity of Lewy bodies, in the brainstem of patients with PD or dementia with Lewy bodies.[110] However, no association was observed between immunostaining and either DNA breaks or activated caspase 3. In an analysis of four PD brains, Jellinger did not observe any difference in the expression of c-jun compared to controls.[111]

SUMMARY AND CONCLUSIONS

In summary, there is growing evidence that the diverse molecular pathways of programmed cell death are likely to play a role in the adult-onset neurodegenerative diseases, including PD. Among these pathways, there is much evidence that the MAPK signaling cascade is likely in some contexts to be an important set of mediators, and it is quite likely that c-jun in particular may play a role. It is likely that c-jun is an important mediator of transcriptionally regulated death pathways that are activated by JNK, especially in contexts in which inflammation is an important component, as exemplified by JNK regulation of cyclooxygenase-2 expression in the acute MPTP model. However, it seems also likely that nontranscriptionally regulated mechanisms of JNK induction of death mediators may be important in some contexts of neural degeneration, perhaps, for example, in the setting of axonal degeneration, as suggested by the results of Crocker and colleagues[94] showing protection by a c-jun dominant-negative in an axotomy model.

The prospect of providing neuroprotection by inhibition of the MLKs, as upstream activators of the JNKs, therefore, appears to have a clear scientific rationale and is supported by much empirical evidence. However, it is important to recognize that the evidence that the MLKs play a direct upstream role in mediating programmed cell death in vivo derives entirely from pharmacological studies with the MLK inhibitors CEP1347 and CEP11004. Although these compounds clearly have specific effects on the MLKs, it is also possible that they could have effects on other known, or unknown, kinases. For example, there is evidence that CEP1347 not only inhibits the MLKs, but activates two important kinases involved in cell survival: Akt and ERK.[112] Thus, it will be important in future studies in living animals to use genetic techniques, either gene transfer or transgenesis, to attempt to define the role of the MLKs at the molecular level. Such investigations are important for the additional reason that they may point the way to regionally selective gene therapy-based approaches to regulation of death pathways, so as to avoid the potential adverse effects that might theoretically result from the systemic administration of an antiapoptotic. By these and additional pharmacological approaches, the MAPK signaling cascade is likely to be an important therapeutic target.

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